

## SPECIES DELIMITATION USING MULTIPLE SOURCES OF EVIDENCE FROM THE *PINUS STROBIFORMIS*-*PINUS AYACAHUITE* SPECIES COMPLEX

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### Abstract

**Background:** The Trans-Mexican Volcanic Belt (TMVB) in central Mexico is characterized by peaks of high altitude and geologic instability. In this zone, *Pinus strobiformis* and *Pinus ayacahuite* form a contact zone with *Pinus veitchii*. The taxonomical circumscription of white pines in Central Mexico has been unstable, especially regarding the status of *P. veitchii*.

**Questions:** What are the species boundaries of the montane Mexican white pines species complex? Is *Pinus veitchii* a hybrid or an independently evolving lineage?

**Studied species:** *Pinus strobiformis*, *Pinus veitchii* and *Pinus ayacahuite* species complex.

**Study site and dates:** United States of America and Mexico from 2003 to 2022.

**Methods:** We performed multivariate analyses on 10 morphological characters and characterized the climatic niche divergence and the genetic differentiation using SNPs.

**Results:** Our results showed that *P. veitchii* is morphologically similar to *P. strobiformis*, but does not have intermediate morphological values with *P. ayacahuite*. The ecological niche differentiation was not significant. Genetic analyses showed *P. veitchii* as an independent lineage with evidence of admixture with *P. ayacahuite*, suggesting a gene flow but not a hybrid origin.

**Conclusions:** Two of the three lines of evidence support three independent lineages. Environmental information showed niche conservatism, morphology and genetic structure showed differentiation of all three taxa, with a greater morphological similarity between *P. strobiformis* and *P. veitchii*, and genetic analyses recovered evidence of introgression, suggesting a complex demographic history in the Trans Mexican Volcanic Belt.

**Keywords:** integrative taxonomy, Trans Mexican Volcanic Belt, unified species concept, white pines.

### Resumen:

**Antecedentes:** La Faja Volcánica Trans Mexicana (TMVB) se caracteriza por picos de gran altitud e inestabilidad geológica. En esta zona, *Pinus strobiformis* y *Pinus ayacahuite* forman una zona de contacto con *Pinus veitchii*. La circunscripción taxonómica de los pinos blancos en el Centro de México ha sido inestable, especialmente en lo que respecta a *P. veitchii*.

**Preguntas:** ¿Cuáles son los límites de especies del complejo de pinos blancos mexicanos? ¿Es *Pinus veitchii* un híbrido o un linaje independiente?

**Especies estudiadas:** Complejo de especies *Pinus strobiformis*, *Pinus veitchii* y *Pinus ayacahuite*.

**Lugar y fechas del estudio:** Estados Unidos de América y México de 2003 a 2022.

**Métodos:** Realizamos análisis multivariados de 10 caracteres morfológicos y caracterizamos la divergencia de nichos climáticos y la diferenciación genética utilizando SNPs.

**Resultados:** Nuestros resultados mostraron que *P. veitchii* es morfológicamente similar a *P. strobiformis*, pero no parece tener valores morfológicos intermedios con *P. ayacahuite*. La diferenciación de nichos ecológicos entre especies no fue significativa. Los análisis genéticos mostraron a *P. veitchii* como un linaje independiente con evidencia de introgresión con *P. ayacahuite*, lo que sugiere flujo génico pero no origen híbrido.

**Conclusiones:** Dos de las tres líneas de evidencia respaldan tres linajes independientes. La información ambiental mostró conservadurismo de nicho, la morfología y la estructura genética mostraron diferenciación de los tres taxones, y los análisis genéticos recuperaron cierta mezcla entre las poblaciones de *P. veitchii* y *P. ayacahuite*, lo que sugiere una compleja historia demográfica en la Faja Volcánica Trans Mexicana.

**Palabras clave:** Concepto Unificado de Especie, Faja Volcánica Trans-Mexicana, pinos blancos, Taxonomía integrativa.

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**P**ines have traditionally been identified and classified using morphological and anatomical characters from ovulated cones and leaves (Mergen *et al.* 1965, Hicks 1973, Abbott 1974, Xing *et al.* 2014). Additionally, morphometric studies have been used to identify species limits in taxa with a wide geographic range and high morphological variation (Stead 1983, Matos 1995, Callaham 2013, Xing *et al.* 2014). Some studies have been successful (Abbott 1974, Xing *et al.* 2014), but some others have struggled with high variation among individuals and phenotypic plasticity (Pooler *et al.* 2002, Tauer *et al.* 2012).

There is ample literature on the phenotypic variation patterns along environmental gradients such as temperature and precipitation (Savolainen *et al.* 2007, Aparicio-Rentería *et al.* 2020, Leal-Sáenz *et al.* 2020). However, morphological characters not always respond to geographical or environmental variables in a linear manner (Malusa 1992, Cruz-Nicolás *et al.* 2020), but are the product of the interaction of local adaptation to microenvironments, gene flow, genetic drift, and population history (Borazan & Babaç 2003, Cruz-Nicolás *et al.* 2020, Uribe-Salas *et al.* 2008, Zúñiga *et al.* 2009). In such scenarios, species respond to those environmental challenges by maintaining high levels of genetic diversity and/or through phenotypic plasticity, in particular when individuals are long lived (Cavender-Bares & Ramírez-Valiente 2017).

Conifers tend to lack marked morphological differences in part due to incomplete reproductive barriers that allow for interspecific gene flow (Rehfeldt 1999, Carney *et al.* 2000, Gompert *et al.* 2012, Aguirre-Gutiérrez *et al.* 2015). Natural selection can either maintain the introgressed genes or force barriers to gene flow (Gompert *et al.* 2012), together with extrinsic factors depending on the fitness (Barton & Hewitt 1985, Hamilton & Aiken 2013, Janes & Hamilton 2017). Therefore, conifer species complexes represent a valuable system to test integrative taxonomy methods in order to delimit species and understand how diversity in populations is maintained.

*Pinus ayacahuite* Ehrenb. ex Schldl. and *Pinus strobiformis* Engelm. are two species from the subgenus *Strobus* (Eckenwalder 2009), section *Quinquefolia*, subsection *Strobus* (Syring *et al.* 2007) which constitutes a species complex with similar morphological characteristics which has resulted in multiple synonyms and difficult identification (Kral 1993, Farjon & Styles 1997, Farjon 1998, Maya 2006, Eckenwalder 2009). Among the diagnostic characters that have been used for identification are the seed size, length of the seed wing, needle length, distribution, number of stomata, and seed cone morphology (Pérez de la Rosa 1993, Farjon & Styles 1997, Frankis 2009). Pérez de la Rosa (1993) examined 17 characters of the *P. strobiformis*-*P. ayacahuite* complex and found a pattern of clinal variation in the needle and seed wing length from North (*P. strobiformis*) to South (*P. ayacahuite*), with intermediate forms found in the Trans Mexican Volcanic Belt (TMVB). This result is supported by nuclear genetic markers that find evidence of introgression mostly in the populations of the TMVB (Moreno-Letelier *et al.* 2013, Moreno-Letelier & Barraclough 2015). This area is where *Pinus veitchii* Roehl has been described in temperate and humid mixed conifer forests between 2,600-3,100. Unlike the other two taxa, it has an extremely restricted distribution with fragmented stands in the states of Michoacán, Tlaxcala, Estado de México, and Morelos. *P. veitchii* was described from a population near San Rafael Tlalmanalco, on the slopes of the Iztaccíhuatl volcano, and it is also common also around the Popocatepetl volcano. Perry (1991) also described populations in Tlaxcala, Michoacán and around Mexico City. Farjon & Styles (1997) consider the same three populations, adding one in the Cerro El Zamorano in Guanajuato and another near the Zempoala lake in the state of Morelos, but lists the Michoacán population as *P. strobiformis*. The later shows that depending on the author, the populations of white pines from the TMVB can be assigned to either *P. veitchii*, *P. ayacahuite*, and even *P. strobiformis*, generating confusion among people doing conservation, reforestation efforts, and even Christmas tree production. The impact of not knowing the true circumscription of *P. veitchii* also makes it difficult to ascertain the true distribution of the species, which has caused that previous potential distribution models to either ignore the taxon all together (Laughlin *et al.* 2011, Moreno-Letelier *et al.* 2013, Shirk *et al.* 2018) or possibly overestimate its distribution (Aguirre-Gutiérrez *et al.* 2015). It is important to clarify the circumscription of *P. veitchii*, distribution and genetic relationships with the other Mexican white pines in order to adequately evaluate its conservation status and preserve local adaptations (Boluda *et al.* 2021).

*Integrative taxonomy.* Accurate identification and species delimitation of pine species is important for systematics, forestry, and conservation. A lot of work has been done at incorporating different sources of information to recognize species (Dayrat 2005, Padial *et al.* 2010). These approaches rely on a unified species concept: independently evolving lineages of metapopulations (De Queiroz 2007), which considers all sources of evidence as contributing to the identification of those lineages, without a priori considerations of what kind of evidence should prevail. In this context, it is important to re-evaluate how well morphological, genetic and environmental characters can differentiate the three taxa.

The high morphological variation and taxonomic controversies of the *P. strobiformis*-*P. veitchii*-*P. ayacahuite* species complex makes these taxa a good model to test how morphological and ecological characters help distinguish different lineages, particularly the ambiguous taxonomic position of *P. veitchii* in the TMVB. Our hypothesis is that *P. veitchii* is in a contact zone between *P. strobiformis* and *P. ayacahuite*, and therefore will have intermediate values in morphological, environmental, and genetic variables suggesting a hybrid origin. Our aim is to analyze the species complex using an integrative approach with morphological, ecological and genetic sources of information, based on the unified species complex.

## Materials and methods

*Plant Material.* The plant material was obtained from field samples deposited in the Herbario Nacional MEXU, Instituto de Biología, UNAM (IB-MEXU) and are listed in [Table S1](#). The specimens were evaluated as follows: 62 specimens from the main distribution area of *P. strobiformis*, 11 specimens from *P. ayacahuite*, and 23 specimens previously identified as *P. veitchii* by at least one author from the TMVB, plus one locality of *P. strobiformis* also named *Pinus stylesii* Frankis ex. Businsky ([Table 1](#), Businsky 2008).

*Morphological differentiation among P. strobiformis-P. veitchii-P. ayacahuite species complex.* We used nine morphological and one dendrometric characters which have been used to discriminate species in the genus *Pinus*: Peduncle Width (Wped), Apophysis Length (Lap), Umbo Width (Wum), Seed Length (Ls), Seed Wing Length (Lw), Seed Wing Width (Ww), Needle Length (Lleav), Needle Sheath Width (Wsh), Number of stomatal lines (Ladx) and Tree Height (Treehg; Pérez de la Rosa 1993, Delgado *et al.* 2007). The last character was obtained from information in herbarium collections and field observations.

Leaf measurements were taken from 15 needles taken at random from each individual (n = 99, [Table S1](#)). The needles were fixed in FAA (formaldehyde alcohol, 70 % ethyl alcohol) for observed the stomata lines and counted using a Nikon microscope to 60x in the IB-MEXU (the consulted vouchers are listed in Supplementary [Table S1](#)). Between 2-5 ovulated cones per locality of the three taxa were measured, with 10 scales and seeds from the central part of the cones. A total of 120 cones were measured.

*Statistical analyses.*- We obtained a Pearson correlation matrix of 9 morphological variables using the function “cor” with the corrplot package for R (Kendall 1938, R Core Team 2021, Wei & Simko 2021) and eliminated one of the variables of pairs with a correlation value > 0.8. After that filter, 9 variables were retained ([Figure S1](#)). The 9 variables retained, and the tree height were normalized by standardizing the arithmetic mean values with their z-scores of each one. This was done to ensure that each variable had an equal contribution in the different statistical and multivariate analyses. We used the functions included in the FactoMineR and factoextra packages (Lê *et al.* 2008, Kassambara & Mundt 2017).

We carried out a one-way ANOVA (Analysis of variance) and MANCOVA (Multivariate analysis of covariance) to establish if there are significant differences among the groups: *P. strobiformis*, *P. veitchii* and *P. ayacahuite*, as suggested by Delgado *et al.* (2007). The variables that were significant in a MANOVA (multivariate analysis of variance) analysis were retained for further analyses among groups.

**Table 1.** Populations from the Trans-Mexican Volcanic Belt and adjacent locations with uncertain taxonomic identity.

Site	Perry 1991	Pérez de la Rosa 1993	Farjon & Styles 1997	Farjon 2010	Businský 2008	Frankis 2009	Bisbee 2014
La Palma, Mich.	<i>Pinus ayacahuite</i> var. <i>veitchii</i>	<i>Pinus ayacahuite</i> var. <i>veitchii</i>	<i>Pinus ayacahuite</i>				
San Rafael, Edo. Mex.		<i>Pinus ayacahuite</i> var. <i>veitchii</i>	<i>Pinus ayacahuite</i> var. <i>veitchii</i>				
Texcaltitlán, Edo. Mex.						<i>Pinus strobiformis</i> var. <i>veitchii</i>	
Popocatepetl, San Nicolás de los Ranchos, Edo. Mex.			<i>Pinus ayacahuite</i>	<i>Pinus ayacahuite</i> var. <i>veitchii</i>			
Calchualco, N of Vaqueria, Ver.	<i>Pinus ayacahuite</i> var. <i>veitchii</i>		<i>Pinus ayacahuite</i>				
Cerro Zamorano, Guanajuato		<i>Pinus strobiformis</i>	<i>Pinus ayacahuite</i> var. <i>veitchii</i>				
Talpa y Cuale, Jalisco	<i>Pinus ayacahuite</i> var. <i>veitchii</i>	<i>Pinus strobiformis</i>	<i>Pinus strobiformis</i>				<i>Pinus strobiformis</i>
Cerro del Potosí, NL.			<i>Pinus strobiformis</i>		<i>Pinus stylesii</i>	<i>Pinus stylesii</i>	<i>Pinus stylesii</i>

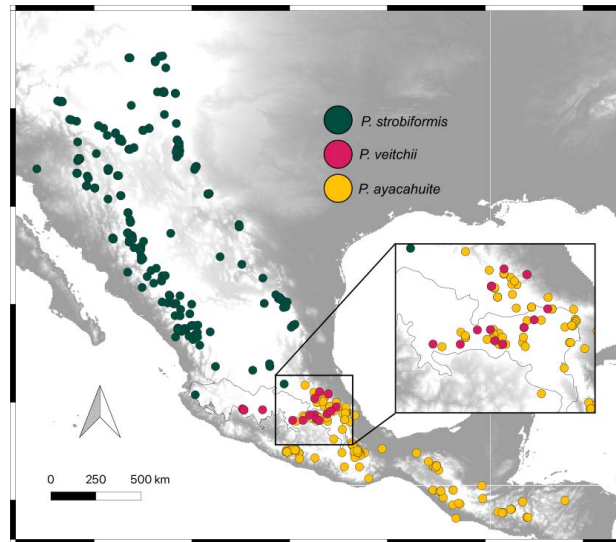
A principal component analysis (PCA) was performed to analyze the morphospace of all individuals and to simplify the variance contribution of all variables. We used the FactoMineR and factoextra packages for R (Lê *et al.* 2008, Kassambara & Mundt 2017). All multivariate analyses were performed using R v. 3.4 (R Core Team 2021). The results were visualized using a UPGMA dendrogram (Sokal & Michener 1958) using PC1 and PC2 scores (Gailing *et al.* 2012).

A Linear Discriminant Analysis (LDA) was performed to select the morphological variables that had a greater contribution to the variance, using the highest PCA eigenvalues (>1) as a guide (Cabrera-Toledo *et al.* 2020). The LDA was used to assign individuals to any of the three groups (*P. strobiformis*, *P. veitchii* and *P. ayacahuite*). The classification success was estimated with a leave-one out cross validation (Nuzzo *et al.* 2022). The results are presented in a confusion matrix with the observed and predicted classifications (Cabrera-Toledo *et al.* 2020, Nuzzo *et al.* 2022).

*Ecological divergence of the P. strobiformis-P. veitchii-P. ayacahuite species complex.* The presence points of the *P. strobiformis-P. veitchii-P. ayacahuite* species complex were compiled from herbaria (IB-MEXU, CIIDIR-DURANGO, FCME of UNAM and UAMIZ-UAM), our own collections and the Global Biodiversity Information Facility (GBIF 2024). The following filters were applied to ensure reliable geographical information (Martínez-Méndez *et al.* 2016): 1) elimination of duplicate records and those with incomplete information, 2) elimination of inaccurate coordinates or those with atypical geographic locations (outside distribution range, cities, water bodies), 3) all records closer than 5 km were eliminated to avoid spatial autocorrelation using the Wallace package (Kass *et al.* 2018). The

total number of records used for further analyses were: *P. strobiformis* (135 records), *P. veitchii* (13 records), and *P. ayacahuite* (85 records, [Figure 1](#)).

The environmental variables used were the 19 bioclimatic variables from WorldClim (Fick & Hijmans 2017). The resolution of all layers was 2.5 min. We used the Variation Inflation Factor (VIF, Brauner & Shacham 1998) to determine how variance is increased in a regression due to multicollinearity. Variables with values over 10 were discarded (James *et al.* 2014, Bruce & Bruce 2017). A one-way ANOVA and a Principal Component Analysis were performed on the retained variables to identify significant differences among species. All analyses were performed with the package FactoExtra and FactoMineR for R v. 3.4 (R Core Team 2021).



**Figure 1.** Distribution of the *Pinus strobiformis*-*Pinus ayacahuite* species complex. Dots represent the distribution of specimens (IB-MEXU and FCME of UNAM, and CIIDIR-Durango), field collections and GBIF data.

Based on the VIF multicollinearity analysis, the variables that were kept (Values <10) for the ENMs were: Average diurnal temperature range (Bio1), Mean Diurnal Range (Bio2), Isothermality (Bio3), Max Temperature of the Warmest Month (Bio5), Temperature Annual Range (Bio7), Mean Temperature of Driest Quarter (Bio 9), Annual Precipitation (Bio12), Precipitation of Driest Month (Bio14), Precipitation Seasonality (Bio15) and Precipitation of Coldest Quarter (Bio19). A Principal Component Analysis was performed with the selected variables to visualize the distribution and differentiation of the taxa.

The ecological niche of each taxon was modeled independently using the MaxEnt algorithm v. 3.3.3 (Phillips *et al.* 2006) implemented by Wallace (Kass *et al.* 2018). The estimated accessible area (M) was defined by a 1-degree buffer around presence points, and model validation was performed using a Jackknife method of non-spatial partition. The following models were tested L, LQ, H, LQH and LQHP, with regularization multipliers from 0.5 to 2.5. The best model was selected using the Akaike Information Criterion also implemented by Wallace. Final maps were displayed using the lower 10th-percentile of training presence. All rasters were visualized with QGIS v. 3.28 (QGIS Development Team 2009 [qgis.org](http://qgis.org)).

The niche similarity was evaluated using three pairs of taxa: 1: *P. strobiformis* vs. *P. ayacahuite*, 2: *P. strobiformis* vs. *P. veitchii*, and 3: *P. ayacahuite* vs. *P. veitchii*. Ecological niche similarity was estimated with Hellinger's I and Schoener's D using ENMTools v. 1.4 (Warren *et al.* 2010). Hellinger's I and Schoener's D range from 0 to 1 (complete divergence to complete overlap). To evaluate the significance of the niche similarity value, then we performed the Background test with ENMTools v. 1.4 (Warren *et al.* 2010). This test compares the niche similarity of a target species (A) with the simulated range of a species (B) as if it were distributed at random in the geographical space.



This simulated range with 100 pseudoreplicates, using a regularization parameter of 2 and a logistic output. The geographical space or background was defined by using a mask that represents the available geographical area for A and B. In our analysis, the mask was constructed using observed presence points and drawing a buffer of 1 min around them and merging the areas. This method of constructing the mask works well for species with well-known distribution and sampling (Warren *et al.* 2010). The buffers and mask were constructed using QGIS v. 3.28 (QGIS Development Team 2009 [qgis.org](https://qgis.org)). For the null distribution, the presence points were generated at random by ENMtools based on the observed presence points of each species. If the observed value of  $I$  between A and B is greater than the null distribution, the ecological niche is more similar than expected by chance. If  $I$  between A and B is smaller than the null distribution, there is niche divergence. Values that fall within the range of the null distribution show that the values of  $I$  are not different from what is expected by chance (Warren *et al.* 2008).

**Genetic analyses.** For the genetic analyses, a total of 96 individuals were collected from the species complex: 31 from *P. strobiformis*, 29 from *P. veitchii* and 35 from *P. ayacahuite* (Fig. S2; Table S2). DNA was extracted from needle tissue using a standard 2X CTAB method (Doyle & Doyle 1987). The DNA was quantified using Qbit and sequenced using a GBS method digested with ApeK1 and sequenced using NovaSeq6000. The sequencing was pair-end with a fragment size of 150 bp.

Data processing was performed as follows: BCFtools mpileup and call commands (v. 1.10.2) were used to call variants from sorted BAM files with no-BAQ, minimum mapping quality and minimum base quality of 20 (Li *et al.* 2009). Reads were mapped to the *Pinus lambertiana* Douglas genome assembly (González-Ibeas *et al.* 2016), a closely related white pine species which was downloaded from TreeGenes, platform that curates genomic information (<https://treegenesdb.org/org/Pinus-lambertiana> and NCBI (*Pinus lambertiana*, BioProject (PRJNA31033, Cronn *et al.* 2008)). BCFtools view was used to select only polymorphic SNPs. VCFtools was used to filter indels and individuals with missing data (Danecek *et al.* 2011). Only biallelic SNPs genotyped across all samples (--max-missing 1) with minimum mean depth greater than 10, minor allele frequency greater than 0.05, and minimum quality score > 25 were selected. Finally, individuals with more than 70 % missing data and loci with more than 50 % missing data were eliminated. The final dataset contained 82 individuals (30 for *P. strobiformis*, 23 for *P. veitchii* and 29 for *P. ayacahuite*), and a total of 1,476 SNPs. A PCA and an Identity by State (IBS) distance analysis were performed with Tassel 5 (Bradbury *et al.* 2007). From the IBS distance matrix we obtained a NeighborJoining unrooted tree visualized with FigTree v. 1.4.4 ([tree.bio.ed.ac.uk](https://tree.bio.ed.ac.uk)).

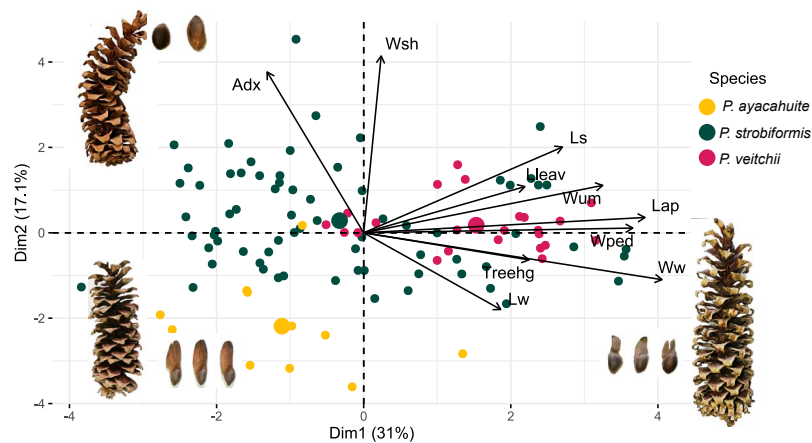
To estimate the admixture proportions between species, VCF file was pruned of SNPs under high linkage disequilibrium with the snpGdsLDpruning function of the SNPrelate package with a threshold of 0.2 and converted to an ordinary PLINK file (.ped) using PLINK v. 1.9 (Purcell *et al.* 2007, Zheng *et al.* 2012). The ADMIXTURE program (v. 1.3.0) was employed to carry out 10 independent runs for K values ranging from 1 to 10 and to evaluate the K value with the lowest cross-validation error (Alexander *et al.* 2009). CLUMPAK v. 1.1 was used to produce bar plots (Kopelman *et al.* 2015).

## Results

**Morphological variation.** The measurements of 99 individuals were split as follows: 65 individuals of *P. strobiformis*, 24 individuals of *P. veitchii* and 10 individuals of *P. ayacahuite*. There were significant differences among the three groups with all variables as shown in the one-way ANOVA analysis (Table S3). Six variables were highly significant ( $P < 0.001$ ): peduncle width, apophysis length, seed length, umbo width, seed wing width, needle sheath width. Two variables were significant at  $P < 0.01$ : needle length and tree height. Two variables were significant at  $P < 0.05$ : seed wing length and number of stomatal lines. Of all variables, only seed wing length showed intermediate values in *P. veitchii* (0.046 mm, s.d. 0.275) when compared with *P. strobiformis* (-0.138 mm, s.d. 0.098) and *P. ayacahuite* (0.787, s.d. 0.283, Table S3). The MANOVA analysis was significant for all variables (Table S4) as well as the MANCOVA (Table S5).

The PCA showed that the PC1 and PC2 explained 29.7 and 16.9 % of the variance, respectively (Figure 2). The morphological variables with the highest contribution to the PC1 were: seed wing width (22.029), apophysis length (18.970), peduncle width (17.520) and umbo width (12.873). For PC2 the variables with the highest contributions were: needles sheath width (38.088), number of stomatal lines (26.581), seed length (13.455) and seed wing length (10.069, Table S6). Seed length and apophysis length were the only two variables that were more similar between *P. strobiformis* and *P. veitchii*.

The scatterplot between PC1 and PC2 shows individuals of the TMVB (identified as *P. veitchii*) are intermingled with individuals of *P. strobiformis* and are not intermediate with *P. ayacahuite* (Figure 2). Loading contributions for each variable can be seen in Table S6. The graphical representation of this pattern can be seen in the UPGMA dendrogram in Figure S3.



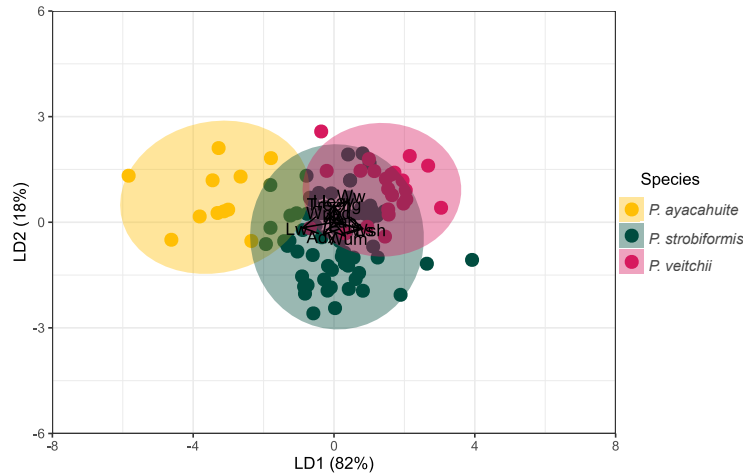
**Figure 2.** PCA of the 10 morphological characters measured in each individual. Dots in shades of green correspond to populations described as *Pinus strobiformis*, shades of blue correspond to populations described as *Pinus veitchii* and in red to *Pinus ayacahuite*.

The Linear Discriminant Analysis explained 100 % of the variation of the original dataset (Figure 3). The variables with the highest discrimination coefficient from LD1 were needle sheath width (Wsh), seed length (Ls) and umbo width (Wum). The variables with the highest discrimination coefficients in LD2 were: needle length, seed wing width and tree height. The plot of LD1 vs. LD2 showed that individuals of *P. strobiformis* and *P. veitchii*, show significant overlap in the ordination space in a 95 % confidence (Figure 3). However, classification analysis through LDA with morphological characters showed that only 12.12 % in total individuals were misclassified, for example, of the 24 individuals of *P. veitchii*, only 5 were misclassified as *P. strobiformis* and 1 as *P. ayacahuite* (Table 2), while 7 % of individuals from *P. strobiformis* were classified as *P. veitchii*. All but one of the samples from *P. ayacahuite* were assigned correctly (90 %; Table 2).

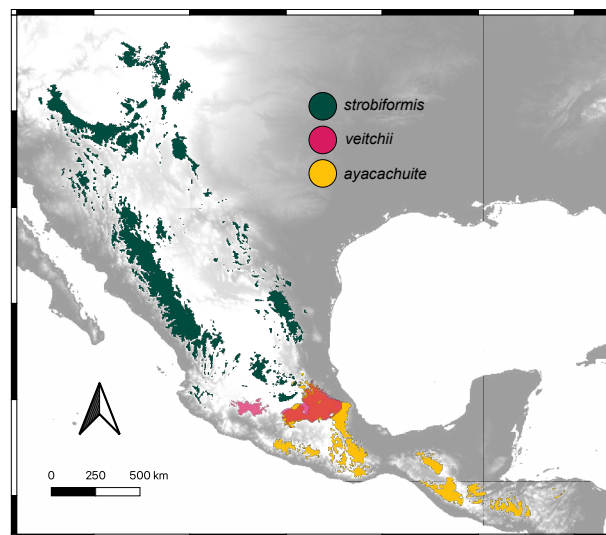
*Ecological niche models and environmental differentiation.* The one-way ANOVA of the retained environmental variables found highly significant ( $P \leq 0.01$ ) differences among species for 9 variables: mean diurnal range (Bio2), isothermality (Bio3), maximum temperature of the warmest month (Bio5), mean temperature of the driest quarter (Bio9), precipitation of the warmest quarter (Bio18), precipitation of the coldest quarter (Bio19). Precipitation seasonality (Bio15) was moderately significant ( $P = 0.015$ ) and precipitation of the driest month was not significant (Table S7). In the PCA the first component (PC1) explains 32.6 % of the variance, PC2 the 27.9 % of variance. No clear differentiation among groups was observed (Figure S4).

The ENM for the three taxa were better than expected by chance and had AUC values  $> 1$ . The best models selected with the AIC were Linear with a RM of 0.5 for *P. veitchii*, LQHP with a RM of 1.5 for *P. strobiformis* and Hinge with a RM of 1.5 for *P. ayacahuite*. The ENMs for *P. strobiformis* and *P. ayacahuite* had overprediction but

were restricted mostly within their known distribution. The model with more overprediction was *P. veitchii*, with a predicted range across most of the TMVB where it only grown in a few isolated populations (Figure 4). The most important environmental variable was isothermality for all three species but in different proportions: *P. strobiformis* (37.9 %), *P. veitchii* (49.8 %) and *P. ayacahuite* (73.3 %). Another variable which was relevant for *P. veitchii* was Temperature annual range (9.5 %), but was not important for *P. strobiformis* nor *P. ayacahuite*. For *P. strobiformis*, the second most important variable was Maximum Temperature of the Warmest Month (17.5 %), and Precipitation of Coldest Quarter (7.8 %) and for *P. ayacahuite*: Annual Mean Temperature (16.1 %) and Precipitation Seasonality (2.6 %), see supplementary material (Table S8). The niche overlap tests (Hellinger's I and Schoener's D) show niche conservatism for most comparisons, with observed values being higher than expected by chance (Figure 5)



**Figure 3.** Plot of LD1 vs LD2 obtained from morphological variables from *Pinus ayacahuite*, *Pinus strobiformis*, and *Pinus veitchii*.



**Figure 4.** Climatic niche models for the present conditions for *Pinus strobiformis*, *Pinus veitchii*, and *Pinus ayacahuite*.

*Genetic analyses.* The genetic clustering analyses showed a differentiation among all species. In all cases, *P. strobiformis* has recovered as differentiated genetic group (Figure 6A-C), while *P. veitchii* and *P. ayacahuite* showed some degree of overlap (Figure 6A-B) or admixture (Figure 6C). In particular, the admixture analyses showed no differentiation between *P. ayacahuite* and *P. veitchii* with  $K = 2$ , but the genetic structure was later recovered with higher  $K$  values, but with a pattern that can be interpreted as either introgression or incomplete lineage sorting (Figure 6C).



## Discussion

*Species delimitation and integrative taxonomy.* There are several factors which make species delimitation difficult for white pine species of the TMVB. First, the wide distribution along a latitudinal cline of the *P. strobiformis*-*P. veitchii*-*P. ayacahuite* species complex provides a gradual transition of environmental conditions, instead of abrupt geographical barriers. Second, the high morphological variation in *P. strobiformis* could be due to environmental or underlying genetic factors such as local adaptation (Menon *et al.* 2018, Leal-Sáenz *et al.* 2020), or an interaction of both. This lack of clear limits makes it important to use a wide arrange of traits that represent the geographical variation of the species complex to be able to adequately identify independent lineages (De Queiroz 2007, Cicero *et al.* 2021).

Morphological traits showed significant differences among all taxa (ANOVA, MANOVA, MANCOVA, [Tables S3-S5](#)), but the PCA and LDA analyses do not show a clear-cut difference between *P. veitchii* and *P. strobiformis* ([Figures 2](#) and [3](#)). However, *P. ayacahuite* is morphologically distinct from the other two taxa. Contrary to our expectations, *P. veitchii* did not have intermediate morphological values, despite having an intermediate distribution and being in a potential contact zone. In other pine populations with known hybrid origins, the hybrids often exhibit intermediate morphological values between the parental species, as it has been described in *P. densata* Masters (Xing *et al.* 2014), hybrids between *P. montezumae* Lamb. and *P. pseudostrobus* Endl. (Delgado *et al.* 2007). In the latter case, hybrids were not consistently assigned to a hybrid cluster with a LDA but were also assigned to their parental species with nearly equal proportions. A similar case has been observed in a hybrid zone between *P. echinata* Hort. ex Carrière and *P. taeda* L., where hybrids were assigned with parental species in high proportion (Chen *et al.* 2004). However, with *P. veitchii*, only 12.5 % of individuals were assigned to *P. strobiformis* ([Table 2](#)).

**Table 2.** Classification analysis of all the individuals in this study, based on LDA results.

Real class	Predicted class (%)		
	<i>P. strobiformis</i>	<i>P. veitchii</i>	<i>P. ayacahuite</i>
<i>P. strobiformis</i>	60 (93)	5 (7)	0
<i>P. veitchii</i>	5 (21)	18 (75)	1 (4)
<i>P. ayacahuite</i>	1 (10)	0	9 (90)

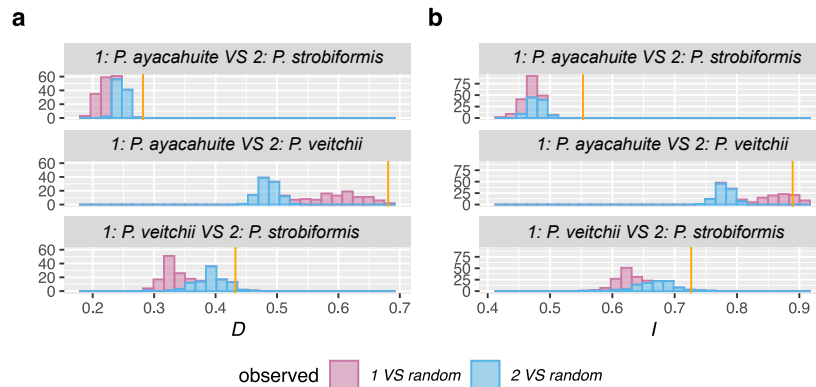
Taxonomic treaties of white pines have classified *P. veitchii* as a variety of *P. ayacahuite* (Farjon 1998, Maya 2006, Leal-Sáenz *et al.* 2020), but Frankis (2009) observed that individuals from the TMVB had seeds and wings 5 to 10 times larger than those of *P. ayacahuite*, making it more similar to *P. strobiformis*, which is consistent with our results. Meanwhile, Businský (2008) has proposed that those populations should be seen as a separate species, *P. veitchii*.

*Environmental differentiation.* The ecological niche models recovered the known distribution for two of the three taxa. The exception was *P. veitchii*, which is known from just a few localities but has a much wider potential distribution ([Figure 4](#)). This could be due to historical or ecological reasons (barriers to dispersal, competition), or due to a higher uncertainty of the models when training with few presence points, leading to overprediction. The environmental variable that is more important for all taxa is isothermality, which is a consequence of the latitudinal cline of the species complex. The higher values of isothermality were seen in *P. ayacahuite*, consistent with a more tropical distribution (Del Castillo *et al.* 2004).

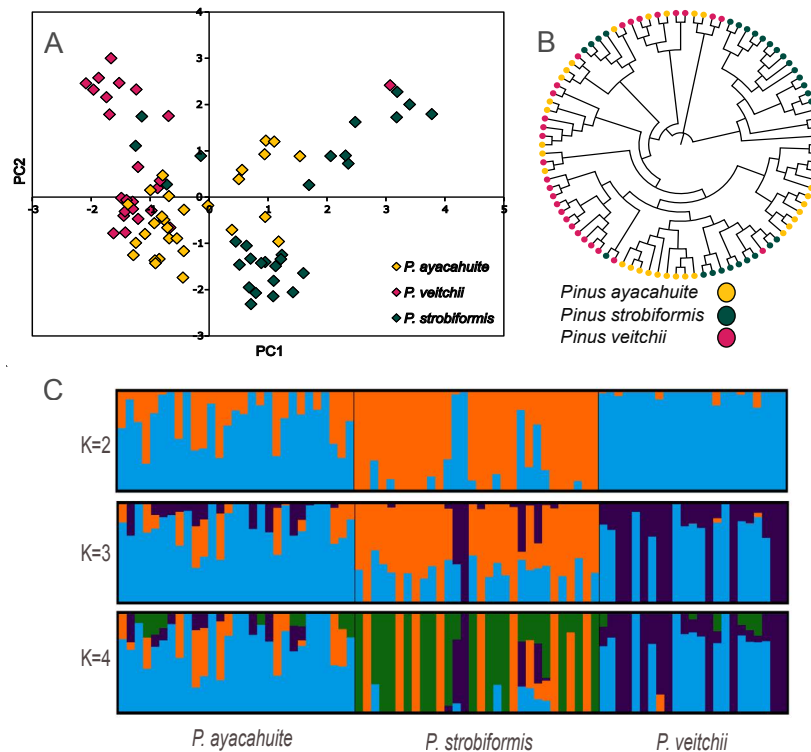
The one-way ANOVA showed a significant differentiation of most variables (the exception was Bio14 Precipitation in the driest month, [Table S7](#)) for the species complex, however, the PCA results show clinal variation rather than clearly defined clusters ([Figure S4](#)). All taxa exhibit overlapped in the environmental space, with *P. veitchii* display-

ing intermediate values. This result is the opposite of what was observed with morphology, where *P. veitchii* was not intermediate, but overlapped with *P. strobiformis* (Figure 4). This result was to be expected due to the geographically intermediate position of *P. veitchii* along a latitudinal cline, but contradicts the clear differentiation between *P. veitchii* and *P. ayacahuite* observed with morphological characters.

The lack of clear clustering in the PCA of environmental variables can be observed in the background test, where all comparisons are either no different to a random distribution or more similar than expected by chance (Figure 5). This result coincides with previous studies where *P. strobiformis* and *P. ayacahuite* are significantly more similar than expected by chance (Moreno-Letelier *et al.* 2013, Aguirre-Gutiérrez *et al.* 2015). However, as these two taxa are not sister species, the niche conservatism can be explained by shared ancestry and evolution in allopatry (McCormack *et al.* 2009). The case of *P.*



**Figure 5.** Background test results for the *Pinus strobiformis*-*Pinus veitchii*-*Pinus ayacahuite* species complex. The observed I and D values are shown by the yellow bars. The null distributions of a) D and b) I values are shown by the histograms.



**Figure 6.** Genetic differentiation analyses: A) Principal component analyses; B) NJ distance unrooted tree; C) Structure analyses showing different values of K.

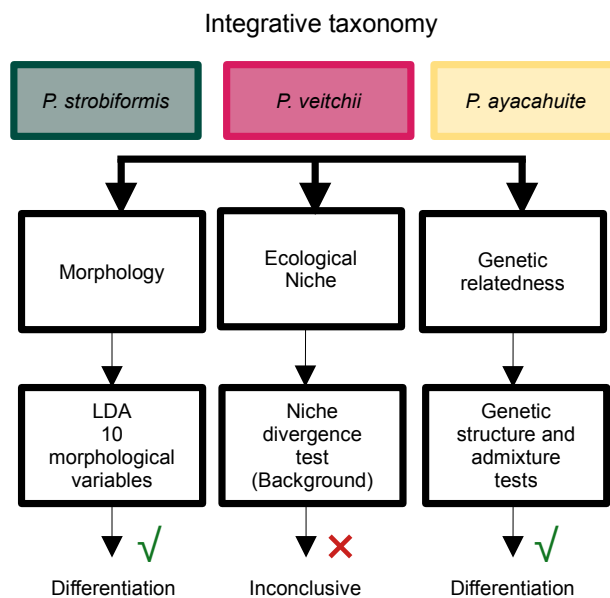
*veitchii* requires more study as it has nested niche with *P. strobiformis* and *P. ayacahuite* (Figure 5) which may hint of incipient peripatric speciation (Moreno-Letelier & Piñero 2009, Moreno-Letelier *et al.* 2013, Castellanos-Morales *et al.* 2016).

Finally, the lack of niche divergence in the three species constitutes evidence against the possibility of a hybrid origin of *P. veitchii*, because in the absence of other reproductive barriers, different environments would prevent back-crossings. The well documented case of hybrid speciation in pines, *Pinus densata*, exhibits significant ecological niche differentiation of the hybrid and parental species, which in turn reinforced the reproductive barriers (Mao & Wang 2011). However, partially overlapping ecological niche observed in Mexican white pines is more suggestive of allopatric and/or peripatric speciation (McCormack *et al.* 2009).

**Genetic analyses.** The genetic analyses showed an opposite pattern to that observed with morphological characters. Our results show a closer relationship between *P. veitchii* and *P. ayacahuite* (Figure 6A-C), and a clear differentiation of *P. strobiformis*. However, there is evidence that introgression might have played a role in those similarities. The PCA analysis has an area of overlap between *P. ayacahuite* and *P. veitchii* (Figure 6A), which can be later seen as evidence of gene flow between those two species in the admixture analyses (Figure 6C). However, standard genetic aggregation analyses cannot determine the direction or the age of said gene flow. Therefore, we require further analyses to be able to clarify how speciation occurred and whether the genetic similarities are due to introgression after speciation or are the result of incomplete lineage sorting (Zhou *et al.* 2016).

**Integrative taxonomy.** As mentioned above, integrative taxonomy aims at including multiple lines of evidence for species delimitation (Dayrat 2005). In this study we used the Unified Species Complex (De Queiroz 2007) in order to aid us in this task. Our results show two lines of evidence supporting the existence of three independent lineages: morphology and genetic differentiation (Figure 7), while niche divergence was not conclusive. However, De Queiroz (2007) stated that under the unified species concept the presence of a specific property that separates a lineage is evidence (morphology and genetic structure in our case), while the absence of a property (niche divergence) only means that the lineage has not evolved that property, but it is not evidence against independence.

The *P. strobiformis*-*P. veitchii*-*P. ayacahuite* species complex from a latitudinal cline, going from the Southwestern United States to El Salvador (Lanner 1996). The TMVB, is where the Southern limit of *P. strobiformis* meets with the Northern limit of *P. ayacahuite*. This area has been identified as a biological corridor (Ornelas *et al.* 2013,



**Figure 7.** Summary of the process of species delimitation using integrative taxonomy and a Unified Species Concept (De Queiroz 2007).

Mastretta-Yanes *et al.* 2015) and important for hybridization in several species (Delgado *et al.* 2007, López-Reyes *et al.* 2015). The combined evidence of our study shows that *P. vetichii*, *P. strobiformis* and *P. ayacahuite* constitute independent lineages, which could be considered species in their own right, supporting our hypothesis. However, our results do not support the hybrid origin of *P. vetichii*, because neither the morphological nor genetic analyses show intermediate values, as it has been observed in other pine populations with known hybrid origins (Delgado *et al.* 2007). The genetic information shows a clear differentiation between *P. strobiformis* and *P. vetichii*, with evidence of introgression with *P. ayacahuite*. Therefore, more analyses are required to be able to distinguish between introgression and shared ancestral polymorphism and determine which taxon is the sister species to *P. vetichii* (Zhou *et al.* 2016, Montes *et al.* 2022). We did not identify significant ecological differentiation; however, it is not a requirement to recognize a species under the unified species concept (De Queiroz 2007).

### Supplementary material

Supplemental material for this article can be accessed here <https://doi.org/10.17129/botsci.3364>

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