

# Structure, diversity, and genetic delimitation of two *Magnolia* species endemic to eastern Mexico

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## Abstract

In Mexico, *Magnolia* species of the section *Macrophylla* have been segregated based on phenotypic characteristics. However, the wide environmental variation throughout their distribution area in the eastern region of the country could be the main cause of the high degree of morphological variation among the taxa of this section. Therefore, it is necessary to carry out studies at the genetic level to confirm the taxonomic identity of these species. In the present study, the diversity and genetic structure of the populations of *Magnolia rzedowskiana* and *M. zotictla* were estimated using simple sequence repeat markers. We evaluated whether the taxonomic delimitation as independent species is consistent with their degree of genetic differentiation, comparing three regions of chloroplast DNA: trnH-psbA, ORF350, and rpl32-trnL. The DNA was obtained from four populations of the two species, which are distributed in the states of Hidalgo, Querétaro and San Luis Potosí. The populations presented intermediate-high genetic diversity ( $H_e = 0.52-0.60$ ), limited gene flow ( $N_m = 0.62$ ) and high genetic differentiation ( $F_{st} = 0.288$ ), which may be related to their low density and strong geographic isolation, caused by the high degree of fragmentation of their habitat. The phylogenetic analysis and the genetic differentiation values indicate that each of the populations of the two *Magnolia* species analyzed behaves as an independent evolutionary unit, so the taxonomic delimitation of the three *M. rzedowskiana* populations must be reconsidered. The results obtained could be very useful for implementing management and conservation strategies for these populations in the short term.

**Keywords:** Chloroplast DNA, *Macrophylla* Section, *Magnoliaceae*, Molecular markers, Population genetics, Taxonomic delimitation

## Introduction

The family Magnoliaceae includes about 330 species with distributions in temperate and tropical regions of America and Asia (Kim & Suh, 2013; Vázquez-García et al., 2015; Linsky et al., 2022). It is a group of great biological interest, due to its role in helping us understand how the first flowering plants emerged. In addition, magnolias have ecological and ethnobotanical importance for their food, medicinal, and ornamental uses (Fan et al., 2020; Linsky et al., 2022).

Wang et al. (2020) recognize 15 sections in the genus *Magnolia*, of which the *Macrophylla* section in particular has nine species, two distributed in Canada and the United States of America (Azuma et al., 2001; García-Morales et al., 2017), and seven in Mexico. In the last decade, new species of the section *Macrophylla* have been described for Mexico. From *Magnolia dealbata* Zucc. (with distribution restricted to the north of Oaxaca), six species were segregated, which are distributed along the eastern slopes of the country: *Magnolia vovidesii* A. Vázquez, Domínguez-Yescas & L. Carvajal in the center of Veracruz (Vázquez-García et al., 2013); *M. rzedowskiana* A. Vázquez, R. Domínguez & R. Pedraza in Hidalgo, Querétaro and San Luis Potosí (Vázquez-García et al., 2015); *M. nuevoleonensis* A. Vázquez & Domínguez-Yescas in Nuevo León (Vázquez-García et

al., 2016); *M. alejandrae* García-Mor. & Iamónico in Tamaulipas (García-Morales et al., 2017); *M. zotictla* A.Sánchez-Gonz., Gut.-Lozano & A. Vázquez in Hidalgo and Puebla (Sánchez-González et al., 2021); and *M. mixteca* A. Vázquez & Domínguez-Yescas in Oaxaca (Vázquez-García et al., 2021).

The seven species of this complex have been described and segregated based on phenotypic characteristics (general morphology) and their geographical distributions. However, variation in the environmental conditions in which they grow can affect the expression of phenotypic attributes and increase divergence due to plasticity (Coates et al., 2018). Therefore, it is necessary to carry out studies at the genetic level to confirm the taxonomic identity of the species (Chávez-Cortázar et al., 2021).

*Magnolia* species from the *Macrophylla* section are considered at risk of extinction (Gutiérrez-Lozano et al., 2020; Chávez-Cortázar et al., 2021), mainly due to changes in land use and the indiscriminate extraction of their flowers and fruits for use by the local population (Vázquez-García et al., 2015). A common pattern in *Magnolia* species worldwide is the reduction in population genetic variability, mainly due to habitat fragmentation and loss. The reduction in the size of their populations and reproductive (geographical) isolation has led to inbreeding and genetic drift processes (Tamaki et al., 2016; Galván-Hernández et al., 2020; Muñiz-Castro et al., 2020; Linsky et al., 2022; Gutiérrez-Lozano et al., 2023).

The use of microsatellite markers or simple sequence repeats (SSR) to estimate the structure and genetic diversity in *Magnolia* and other plant genera is widespread, due to the method's properties of codominance, high polymorphism, and reproducibility and efficiency, among others (Fan et al., 2020; Rico et al., 2021). Some representative studies include the following species: *M. stellata* in Japan (Tamaki et al., 2016); *M. officinalis* (Yu et al., 2011), and *M. wufengensis* (Chen et al., 2014) in China; and four more species from the *Talauma* section (Testé et al., 2022) in Cuba. In Mexico, molecular markers of this type have been used in *M. sharpii* in Chiapas and *M. schiedeana* in Veracruz (Newton et al., 2008), in *M. mexicana* in Puebla and Veracruz (Gutiérrez-Lozano et al., 2023), for the analysis of the *Magnolia pacifica* complex in Jalisco (Muñiz-Castro et al., 2020), and in five species of the *Macrophylla* section present in Mexico (Chávez-Cortázar et al., 2021).

Reliable taxonomic delimitation of species is essential in various areas of biology (ecology, biogeography, evolution, and systematics, among others), and for the development of management and conservation programs. In taxa with recent evolutionary divergence, their delimitation may be more complex, as is the case of several species complexes in *Magnolia*. DNA sequencing of different chloroplast and/or nuclear regions in several complexes of *Magnolia* species from America and Asia has enabled their taxonomic identity to be identified with greater certainty and hypotheses to be generated about their phylogenetic relationships and natural history (Budd et al., 2015; Tamaki et al., 2018; Rico et al., 2021; Testé et al., 2022).

Using microsatellite markers, Chávez-Cortázar et al. (2021) carried out a study of five of the seven species of the *Macrophylla* section that are distributed in Mexico, with the purpose

of determining whether the taxonomic delimitation carried out based on basic morphological characters coincided with the data obtained at the genetic level. However, their results were not conclusive, due to a limited number of samples and markers, so they consider that it is important to use more appropriate molecular markers (genetic sequences of chloroplasts and nucleus) and a more complete sampling that covers the entire range of distribution of species, to elucidate the number of existing taxonomic units. Testé et al. (2022) suggest that the most appropriate strategy to distinguish between *Magnolia* taxa can be developed by integrating different disciplines such as biogeography, ecology, morphology and molecular biology.

The species *Magnolia rzedowskiana* and *M. zotictla* present temporary reproductive isolation, despite their geographical proximity, so it is likely that their evolutionary divergence is recent. Analyses of variation in ecological, phenological, morphological (Vázquez-García et al., 2016; Gutiérrez-Lozano et al., 2020; Sánchez-González et al., 2022), and anatomical characteristics (Cuapio-Hernández, 2022) have enabled the populations of the two species to be clearly differentiated from each other. However, it is important to corroborate their taxonomic identity with studies at the genetic level, as a direct source of information on processes related to speciation. Genetic variation is completely heritable, unlike phenotypic variation, which has an environmental component (Coates et al., 2018).

Therefore, the objectives of the present study were the following: 1) to analyze, based on SSR markers, the structure and genetic diversity of the populations of *M. rzedowskiana* and *M. zotictla* throughout their geographical distribution area; 2) to estimate a relationship between genetic distance and geographic distance between populations if it exists; that is, the effect of variation in local environmental conditions with distance; and 3) to evaluate whether the separation between the two taxa, defined in previous studies based on morphological, anatomical, geographical, and phenological differences, is consistent with the degree of genetic differentiation of the populations, using three regions of chloroplast DNA as markers. The results obtained could be very useful for implementing appropriate management and conservation strategies in the two *Magnolia* species in the short term.

## Material and methods

### Study area

The populations of *M. rzedowskiana* and *M. zotictla* are distributed in the cloud forest (CF) of the Sierra Madre Oriental (Figure 1). The climate in the study area is temperate with rain all year round C (f), with the presence of fog, so the degree of humidity is high, ranging between 55 and 65 %. The average annual temperature ranges between 17.1 and 20.7 °C and the total annual precipitation is between 1,048 and 2,385 mm. The elevational distribution interval of the CF varies from 600 to 2,500 m (Rosas et al., 2019), but the populations analyzed are distributed between 1,364 and 1,914 m asl. The localities

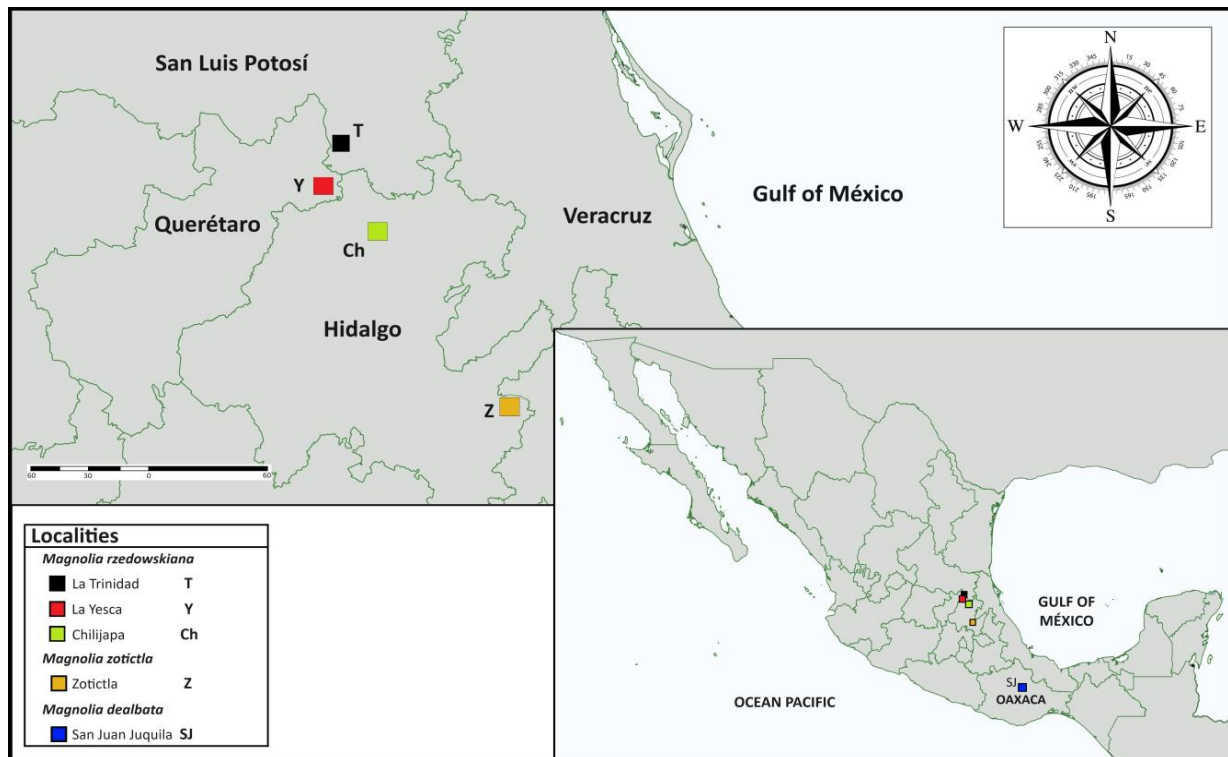


Figure 1  
Distribution map of natural populations of *M. rzedowskiana* and *M. zotictla* species in Mexico

Table 1  
Geographic data of the localities where leaf samples of the three *Magnolia* species were collected. The species *M. dealbata* was included in the analysis only as a reference or external group.

| Locality                     | Municipality           | State           | Latitude     | Longitude    | Elevation (m) |
|------------------------------|------------------------|-----------------|--------------|--------------|---------------|
| <i>Magnolia rzedowskiana</i> |                        |                 |              |              |               |
| 1. Chilijapa (Ch)            | Tepehuacán de Guerrero | Hidalgo         | 21° 0.275'N  | 98° 51.780'W | 1364          |
| 2. La Yesca (Y)              | Landa de Matamoros     | Querétaro       | 21° 13.058'N | 99° 7.949'W  | 1716          |
| 3. La Trinidad (T)           | Xilitla                | San Luis Potosí | 21° 24.656'N | 99° 4.229'W  | 1914          |
| <i>Magnolia zotictla</i>     |                        |                 |              |              |               |
| 4. Zotictla (Z)              | Acaxochitlán           | Hidalgo         | 20° 13.519'N | 98° 9.867'W  | 1743          |
| <i>Magnolia dealbata</i>     |                        |                 |              |              |               |
| 5. San Juan (SJ)             | San Juan               | Oaxaca          | 17° 20.427'N | 96° 17.813'W | 1672          |

where the populations of *M. rzedowskiana* are distributed are 1. Chilijapa (Tepehuacán de Guerrero), Hidalgo; 2. La Yesca (Landa de Matamoros), Querétaro; and 3. La Trinidad (Xilitla), San Luis Potosí. The population of *M. zotictla* is found in 4. Zotictla (Acaxochitlán), Hidalgo (Figure 1, Table 1).

During July and August 2022, leaf tissue was collected from 10 to 30 trees per location, depending on the size of the population and the distance between individuals, since in some sites they had a grouped distribution. For the collection of leaf samples, the average distance between sampled trees was at least 50 m, to avoid inbreeding effects. The samples obtained were placed in plastic bags to which silica beads were added for drying and they were stored in a freezer at  $-20^{\circ}\text{C}$  until they were processed in the laboratory. Additionally, leaf

tissue samples were obtained from 15 individuals from a population of *Magnolia dealbata*, which is distributed in San Juan Juquila Vijanos, Oaxaca, Mexico, in order to have an external group of the *Macrophylla* section as a reference in the analysis.

#### DNA extraction and microsatellite amplification

To obtain DNA, the method of Doyle and Doyle (1987) was used, with some modifications, namely the use of liquid nitrogen to macerate the tissue, CTAB 2x-PVP extraction buffer (100 mM Tris-HCl pH 8.0, 1.4 M NaCl, 20 mM EDTA, 2 % hexadecyltrimethylammonium bromide, 0.5 % polyvinylpyrrolidone) with 0.2 % 2-β-mercaptoethanol, and the use of phenol: chloroform:isoamyl (25:24:1) to separate DNA from the rest of the cellular components. The microsatellites were amplified

Table 2

Molecular markers used in the genetic analysis of the three *Magnolia* species. aT: alignment temperature in °C, bp: base pairs.

| Sequence | Marker (5'-3')                                                 | aT (°C) | bp      |
|----------|----------------------------------------------------------------|---------|---------|
| M6D3     | ACA TGG ATA GTC GTT GGA TA<br>ACC CCA CTG AAG ACA AAC AT       | 57      | 85–130  |
| M6D8     | CGA GTG GCA TTT CCG TAA TA<br>GAA CCT GGC GCA CCG TAG TC       | 64      | 130–160 |
| M6D4     | CAC CGT ACC CTA TCA GAA CC<br>ATT TTC AGC ATC ATC AGT TG       | 53      | 120–180 |
| M6D1     | ACT GGA GCA GTG CCT GGA TA<br>TCG CAA CTG CGT GTT CTC AT       | 55      | 80–140  |
| M10D3    | GTC TAG TGA GCC GCA AAT GG<br>GTG AAC AGC TTT CTT GTG AA       | 52      | 190–220 |
| M6D10    | CGA CGA CGA AAC TAC TAA CA<br>TTA AGT TGA GGT GGA ATG AC       | 55      | 135–180 |
| M10D8    | AGC CCT CTA TAC ACG CAC ACA T<br>CGG AGC TAC AAG GAG CAG AAT A | 55      | 200–310 |
| M15D5    | GAT CGT TGC TGG CTC GC<br>GCC GCC TGG ATT ATG AA               | 54      | 100–110 |

with eight primers (SSR, Table 2) developed by Isagi et al. (1999). The mixture for the polymerase chain reaction (PCR) was 1.14 µl of DNA (50 ng/µl), 1.6 µl of PCR buffer (1x), 1.44 µl of MgCl<sub>2</sub> (4.5 mM), 0.16 µl of dNTPs (0.2 mM), 0.64 µl of forward and 0.64 µl of reverse primer (2 µM each), 2.28 µl of sterile double distilled water and 0.1 µl of Taq polymerase (0.2 U, PROMEGA), for a final volume of 8 µl. Amplification was carried out in a gradient thermo cycler (Arktik, Thermo Scientific) with initial denaturation 95 °C for 5 min; 35 cycles starting at 95 °C for 55 seconds, 55 seconds at the alignment temperature of each marker, 50 seconds of extension at 72 °C, and a final extension at 72 °C for 6 min. Amplified samples were separated on 15 % acrylamide gels (acrylamide/bisacrylamide 19:1, 40 % solution, Sigma Aldrich) on 1× TBE buffer at 90 volts for 1.5 hours, stained with 1 % ethidium bromide solution, and visualized in a UVP transilluminator.

**Analysis of data.** With the Gel Analyzer program (Lazar and Lazar, 2010), the size of the bands was recorded to generate a matrix with the number of base pairs per allele, with which the following variation indicators were estimated: average number of alleles per locus (Na), effective number of alleles per locus (Ne), percentage of polymorphisms (%P), observed heterozygosity (Ho), expected heterozygosity (He), Shannon index (I), and fixation coefficient (f). In addition, the following complementary analyses were performed: analysis of molecular variance (AMOVA), and the significance levels were tested with 999 permutations to estimate differences between populations; Hardy-Weinberg equilibrium test (HWE) in each of the populations, applying the chi-square test; estimation of Nei's genetic distances (Nei et al., 1983), with which a neighbor-joining dendrogram was created with a 500-step bootstrap to establish group assignment; and the number of migrants per generation (Nm) was calculated from the paired F<sub>st</sub> values, using the Bonferroni correction for the p value. All these analyses were carried out with the GenAlEx program, v.641 (Peakall and Smouse, 2006).

In addition to differences based on Nei distance values, genetic structure was determined by two complementary methods. First, a Bayesian assignment analysis was carried out, which estimates the probability that an individual is included in a particular genotype within the population of origin, considering K genotypes (from 2 to 10). For this, the STRUCTURE program (Pritchard et al., 2000) was used, with an iteration of 50,000 chains and 50,000 bootstrap resamplings; the analysis was repeated 10 times to establish a confidence interval. To select and visualize the number of genotypic groups between populations, the ΔK value was calculated by the method of Evanno et al. (2005), which enables the model with the maximum rate of change of  $\ln Pr(X|K)$  to be found. This was done with the STRUCTURE SELECTOR web server (Li and Liu, 2018). The second approach uses an integrated and linear differentiation measure, established from a multivariate correlational model, where each marker constitutes a variable to generate a principal component analysis. Subsequently, each component is considered a new linear-orthogonal variable, useful as a basis for estimating the degree of differentiation through a discriminant function analysis of principal components (DFAPC). The variation in the data is independent of the origin, and unlike the Nei distances, this analysis considers all the alleles. Finally, a Mantel test was performed to determine if there is a significant correlation between the genetic distances of Nei (Nei et al., 1983) and the geographical distances between populations. The paired geographic distances between population were obtained using the Google Earth Pro application from the geospatial position registered with eTrex Garmin GPS. The procedure was carried out with the STATISTICA program v. 10 (StatSoft, Inc., 2011).

#### **DNA extraction and amplification of three chloroplast regions**

Two fresh young leaves, free of visible damage, were collected from five individuals per population (Table 1). Three

chloroplast genomic regions were amplified; trnH-psbA, ORF350 and rpl32-trnL, which have been reported to have high percentages of variable and informative sites both for plants in general, and for the Magnoliaceae family specifically (Kim & Suh, 2013). PCR reactions included the following reagents: buffer (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> (1×), MgCl<sub>2</sub> (5 mM), dNTPs (0.2 mM), primers (0.1–0.2 µM), Taq polymerase (0.5 U) and 3–6 µL of DNA in a final volume of 25 µL. The primers (Table 3) were selected based on three studies on Magnoliaceae (Azuma et al., 1999; Kim et al., 2001; Azuma et al., 2011). PCR cycles were carried out in a T100 Thermal Cycler Bio-RAD thermocycler; the thermal profile data for the amplified regions is included in a table as supplementary material. The quantity and quality of the PCR products were qualitatively evaluated by electrophoresis in 2 % agarose gels (125 V for 45 min) and were subsequently sent to the University of Arizona (University of Arizona Genetics Core) in the United States of America, where they were purified and sequenced in the “forward” direction using the Sanger technique.

### Sequence alignment and phylogenetic analyses

The sequences obtained were visualized and edited using the programs FinchTV v1.4.0 (<http://www.geospiza.com>), Bioedit v7.0.5.3 (Hall, 1999), and PhyDE v0.9971 (Müller et al., 2010). They were subsequently aligned using the MUSCLE algorithm implemented in Jalview v2.4 (Waterhouse et al., 2009). Once the sequences were aligned, the jModelTest2 v2.1.9 program (Darriba et al., 2012) was used to select the nucleotide substitution models that best fit the data using the Bayesian (BIC) and Akaike information criteria (AIC).

In addition to the sequences generated in the present study, GenBank sequences from the work of Azuma et al. (2011) and Kim & Suh (2013) were included. The phylogenetic tree was reconstructed with Bayesian inference (BI) using the MrBayes v3.2.7 program (Ronquist et al., 2012); a matrix was constructed with the sequences of the three concatenated genomic regions for each of the individuals using the substitution model that best fit each partition. The parameters used were two simultaneous analyses of 15,000,000 generations with four Monte Carlo Markov chains (MCMC) each and a heating parameter of 0.20. The sampling frequency was every 5000 generations, discarding the first 25 % of the generated tree samples and parameter values (burn-in). In the end, the main statistics were evaluated in Tracer v1.7.1 (Rambaut et al., 2018), corroborating the convergence and stationarity of the Markov chains. The majority rule (50 %) consensus tree was visualized in FigTree v1.4.3 (Rambaut, 2016). Additionally, another phylogenetic reconstruction was carried out using maximum likelihood (ML) with the RAxML v8.2.10 program (Stamatakis, 2014) using the same matrix of concatenated sequences as in the Bayesian analysis, indicating the number of partitions. The model used independently for each partition was GTRGAMMA, which is the only model that implements RAxML; 1000 searches for the best tree with maximum likelihood and 1000 bootstrap replications were carried out.

## Results

### Genetic diversity

Eight microsatellite markers were analyzed in samples from 60 individuals of *M. rzedowskiana* from three localities, and 10 individuals of *M. zotictla* from the only known locality for the species. Both species are distributed in the cloud forest of the Sierra Madre Oriental. Additionally, 15 individuals of *M. dealbata*, a species of the *Macrophylla* section, with well-defined taxonomic identity, obtained from the population of San Juan Juquila Vijanos in the state of Oaxaca, were included in the analysis as a reference or external group (Table 4).

The average number and effective number of alleles per locus ranged from 3.37 to 4.12 and 2.25 to 2.85 respectively in the five populations of the three *Magnolia* species. The population with the highest number of effective alleles was La Trinidad, San Luis Potosí ( $N_e = 2.85$ ), followed by Zotictla (Hidalgo), Chilijapa (Hidalgo) and La Yesca (Querétaro). The same pattern occurred with the values of the Shannon index (Table 4). The populations of La Trinidad, La Yesca and Zotictla presented 100 % polymorphism, and the populations of Chilijapa and San Juan Juquila Vijanos (*M. dealbata*) 87.5 %. The observed heterozygosity values were relatively similar in the *M. rzedowskiana* (0.43–0.64) and *M. zotictla* (0.57) populations, but a lower value (0.39) was estimated in the *M. dealbata* population. The population of Chilijapa presented the highest values of heterozygosity ( $H_o = 0.64 \pm 0.13$ ).

The highest values of the inbreeding coefficient ( $F_{is}$ ) were found in the populations of *M. dealbata* ( $F_{is} = 0.24 \pm 0.17$ ) and *M. rzedowskiana* at La Yesca ( $F_{is} = 0.19 \pm 0.23$ ). The  $F_{is}$  value of *M. zotictla* was low ( $0.08 \pm 0.19$ ) compared to that estimated in the Chilijapa population (*M. rzedowskiana*), which presents exogamy ( $F_{is} = -0.23 \pm 0.19$ ). The analysis of molecular variance (AMOVA) shows statistically significant differences between the analyzed populations ( $F_{st} = 0.288$ ,  $p < 0.001$ ). Genetic differentiation within and between populations was 71.2 % and 28.8 %, respectively; gene flow was less than 1 ( $N_m = 0.62$ ).

The chi-square test results ( $\chi^2 = 21.12 \pm 21.77$ ,  $p < 0.001$ ) suggest that the analyzed populations are not in Hardy-Weinberg equilibrium. This is because the observed allelic frequencies differ from the expected frequencies. Additionally, each population shows a different amount of disequilibrium loci. Specifically, La Yesca has all loci in disequilibrium, Chilijapa and La Trinidad have four, *M. zotictla* has three, and *M. dealbata* has three. The La Trinidad and Zotictla populations (Table 5) presented the lowest differentiation coefficient ( $F_{st} = 0.10$ ), the highest number of migrants per generation ( $N_m = 2.289$ ), and therefore the lowest genetic distance (0.19). The populations with the lowest number of migrants per generation and the highest genetic distance were Chilijapa versus San Juan Juquila Vijanos (0.48, 0.34), Chilijapa versus La Yesca (0.45, 0.36) and Zotictla versus La Yesca (0.54, 0.32). The correlation between Nei's genetic distances and geographical distances was not significant ( $\chi^2 = 0.429$ ,  $r^2 = 0.1092$ ,  $p = 0.512$ ), indicating that there is insufficient evidence to support the presence of an isolation by distance model.

The Bayesian assignment analysis indicates that there are five genetic groups ( $K=5$ , Figure 2), differentially distributed among the populations, with three exclusive groups in *M. rzedowskiana* and one in *M. dealbata*. The population of *M. rzedowskiana* from La Yesca (Landa de Matamoros, Querétaro) presented two genetic groups, one shared with the population of La Trinidad and one that is independent. The population of Chilijapa (Tepehuacán de Guerrero, Hidalgo), which has a particular genetic conformation, showed greater similarity with the population of *M. zotictla* (Zotictla, Acaxochitlán, Hidalgo) than with the others. The population of La Trinidad (*M. rzedowskiana*) contains four of the five genetic groups present in the three species (Figure 2) but is genetically closer to the populations of La Yesca (*M. rzedowskiana*) and Zotictla (*M. zotictla*).

The results of the DFAPC ( $F(16.65): > 3.52; P < 0.001$ ) generally agree with the results obtained in the Bayesian assignment analysis and in the neighbor-joining analysis: the population of La Trinidad (Xilitla) is genetically related to the populations of Chilijapa and La Yesca (*Magnolia rzedowskiana*) and to the population of Zotictla (*M. zotictla*). Individuals from the populations of *M. rzedowskiana* from La Yesca and *M. dealbata* from San Juan Juquila Vijanos (Oaxaca) appear in the scatter diagram as groups partially or as completely independent from the others, respectively (Figure 3).

The final matrix of concatenated sequences included 23 of the 25 sequences obtained, since those that lacked information for one or more genomic regions were excluded. The substitution models that were chosen, as they presented the best fit with the data, were HKY+G for psbA-trnH and ORF350, and HKY for rpl32-trnL. The majority consensus tree resulting from the Bayesian inference analysis and the best tree with maximum likelihood were very similar in topology, so only the Bayesian inference tree is shown, including bootstrap values  $\geq 70\%$  from the maximum likelihood analysis (Figure 4).

The genetic sequences of the *Macrophylla* section specimens analyzed formed a clade with support values of 1 for posterior probability (PP) and 88 % for bootstrap (BS, node A). The analysis recovered three clades with relatively high support values (Figure 4). The first is made up of sequences of *M. rzedowskiana* specimens from the population of La Trinidad (San Luis Potosí), the second by sequences of specimens of *M. rzedowskiana* from La Yesca (Querétaro), and the third contains sequences of specimens of *M. rzedowskiana* (population of Chilijapa, Hidalgo) and *M. zotictla* (Zotictla, Hidalgo). However, within this group two independent subgroups were formed, according to the species to which they belong.

## Discussion

### Genetic diversity

Genetic diversity is essential for populations to be able to adapt to changes in environmental conditions (climate change, pests, habitat fragmentation, or disturbances, among others). Preserving the genetic diversity of a species can increase its

chances of surviving over time (Fan et al., 2020; Chung et al., 2023), which is why genetic diversity is a good indicator of adaptive potential.

In the present study, the populations of the three *Magnolia* species exhibited intermediate genetic diversity values ( $H_e = 0.52\text{--}0.60$ ), compared to those estimated in other species of the genus. For example, they are higher than those calculated in *Michelia* (*Magnolia*) *coriacea* ( $H_e = 0.429\text{--}0.498$ , Zhao et al., 2012), in *Magnolia wufengensis* ( $H_e = 0.145\text{--}0.286$ ; Wang et al., 2017) and in *M. patungensis* ( $H_e = 0.228\text{--}0.376$ , Fan et al., 2020) in China; similar to those estimated in five species from the *Talauma* section in Mexico ( $H_e = 0.52\text{--}0.68$ , Aldaba-Núñez et al., 2021), in nine species of magnolias from the Neotropics ( $H_e = 0.458\text{--}0.712$ ; Veltjen et al., 2019), in *Magnolia mexicana* in Mexico ( $H_e = 0.46\text{--}0.70$ , Gutiérrez Lozano et al., 2023); and lower than the values obtained in *Magnolia kobus* in Japan ( $H_e = 0.723$ , Tamaki et al., 2016), in *M. acuminata* from the United States of America and Canada ( $H_e = 0.828\text{--}0.899$ , Budd et al., 2015) and in *Michelia* (*Magnolia*) *shiluensis* in China ( $H_e = 0.650\text{--}0.825$ , Deng et al., 2020).

The wide variation in genetic diversity values in *Magnolia* has been attributed to various causes, although it is generally considered that species with narrow distribution tend to have lower genetic diversity than those with wide distribution (Fan et al., 2020; Aldaba-Núñez et al., 2021). In addition, species with small populations and that develop in fragmented habitats due to natural and/or anthropogenic causes are expected to present low genetic diversity (Wang et al., 2018; Fan et al., 2020). However, in the populations of *M. rzedowskiana* and *M. zotictla*, intermediate to high genetic diversity values were obtained, even though their populations, small and isolated from each other, are characterized by their restricted distributions and highly fragmented habitat (Vázquez-García et al., 2015; Gutiérrez-Lozano et al., 2020, Sánchez-González et al., 2021).

According to Zhao et al. (2012), some plant species can maintain high genetic diversity because their populations tend to be small and isolated naturally, or because of a recent reduction in the size of their populations. In this case, the surviving individuals are representative samples of larger populations that existed in the past (Wang et al., 2020; Chávez-Cortázar et al., 2021). Budd et al. (2015) and Fan et al. (2020) note that long generation times in tree species can cushion the adverse effects (genetic erosion) caused by recent modification of their environment, so it is likely that the longevity of individuals of *M. rzedowskiana* and *M. zotictla* is related to the estimated intermediate-high levels of genetic diversity.

In Mexico, the cloud forest, habitat of both *Magnolia* species, is an ecosystem with high biodiversity, but also with a high degree of fragmentation, mainly due to changes in land use, which is why it is considered at risk (Rosas et al., 2019). For the reasons mentioned above, although the genetic diversity values of the populations are "adequate," they could decrease drastically in subsequent generations if destruction of the CF continues due to human activities. The value of the inbreeding coefficient in the *M. rzedowskiana* populations ( $F_{is} > 0.19$ ) indicates processes of genetic drift or intense reproduction within

Table 3  
Primers used for each of the amplified chloroplast genomic regions.

| Name        | Sequence (5'-3')        | Reference           |
|-------------|-------------------------|---------------------|
| ORF350F     | GTGACAAATGCAATTATTCA    | Kim et al. (2001)   |
| ORF350R     | TTAGTCAACATTATCTCTTTTCG |                     |
| trnH-psbAF  | CGCATGGTGGATTACAATC     | Azuma et al. (1999) |
| trnH-psbAR  | AGACCTAGCTGCTATCGAAG    |                     |
| rpl32-trnLF | CTGCTTCTTAAGAGCAGCGT    | Azuma et al. (2011) |
| rpl32-trnLR | GGATCCCTTTAGGTCGATA     |                     |

Table 4  
Genetic diversity values in populations of *Magnolia dealbata*, *M. rzedowskiana* and *M. zotictla*. N: number of individuals, Na: average alleles per locus, Ne: effective number of alleles per locus, % P: percentage of polymorphism, I: Shannon index, Ho: observed heterozygosity, He: expected heterozygosity, Fis: Fixation index, SE: standard error.

| Locality                     | N  | Na±SE       | Ne±SE       | % p  | I±SE        | Ho±SE       | He±SE       | Fis±SE       |
|------------------------------|----|-------------|-------------|------|-------------|-------------|-------------|--------------|
| <i>Magnolia rzedowskiana</i> |    |             |             |      |             |             |             |              |
| 1. Chilijapa                 | 10 | 3.37 ± 0.65 | 2.69 ± 0.52 | 87.5 | 0.94 ± 0.19 | 0.64 ± 0.13 | 0.53 ± 0.08 | -0.23 ± 0.19 |
| 2. La Yesca                  | 30 | 3.87 ± 0.61 | 2.25 ± 0.20 | 100  | 0.93 ± 0.10 | 0.43 ± 0.12 | 0.53 ± 0.04 | 0.19 ± 0.23  |
| 3. La Trinidad               | 20 | 4.12 ± 0.66 | 2.85 ± 0.53 | 100  | 1.06 ± 0.17 | 0.50 ± 0.1  | 0.56 ± 0.07 | 0.19 ± 0.14  |
| <i>Magnolia zotictla</i>     |    |             |             |      |             |             |             |              |
| 4. Zotictla                  | 10 | 3.62 ± 0.56 | 2.72 ± 0.32 | 100  | 1.05 ± 0.11 | 0.57 ± 0.12 | 0.60 ± 0.03 | 0.08 ± 0.19  |
| <i>Magnolia dealbata</i>     |    |             |             |      |             |             |             |              |
| 5. San Juan                  | 15 | 3.62 ± 0.65 | 2.55 ± 0.4  | 87.5 | 0.96 ± 0.19 | 0.39 ± 0.12 | 0.52 ± 0.08 | 0.24 ± 0.17  |

Table 5  
Number of migrants per generation (Nm: upper diagonal), and fixation index (Fst: lower diagonal of the matrix) among the populations of the three species of magnolias analyzed. \*P<0.001.

| Locality/<br>population | <i>Magnolia rzedowskiana</i> |          |           | <i>M. zotictla</i> | <i>M. dealbata</i> |
|-------------------------|------------------------------|----------|-----------|--------------------|--------------------|
|                         | La Trinidad                  | La Yesca | Chilijapa | Zotictla           | San Juan           |
| La Trinidad             | –                            | 0.71     | 0.60      | 2.29               | 0.59               |
| La Yesca                | 0.26*                        | –        | 0.45      | 0.54               | 0.54               |
| Chilijapa               | 0.30*                        | 0.36*    | –         | 0.81               | 0.48               |
| Zotictla                | 0.10*                        | 0.32*    | 0.24      | –                  | 0.67               |
| San Juan                | 0.30*                        | 0.32*    | 0.34*     | 0.27*              | –                  |

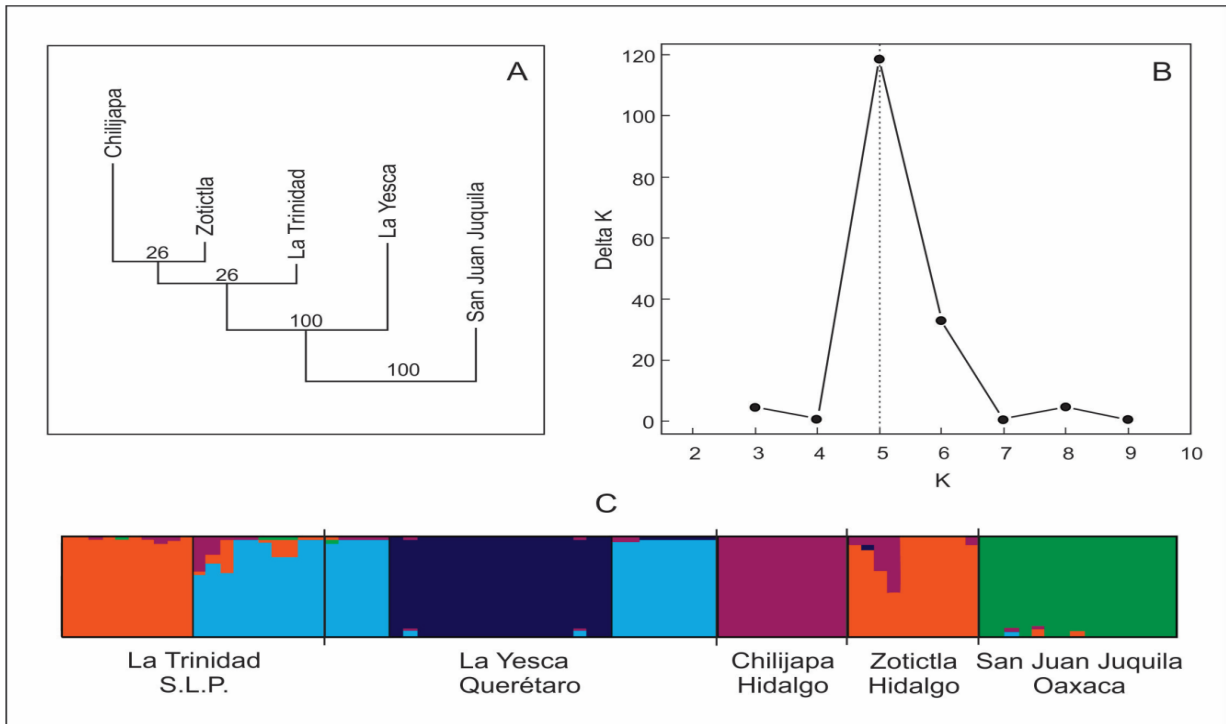


Figure 2

A. Neighbor-joining dendrogram. B. Estimated number of genetic groups (K), derived from STRUCTURE clustering analysis. C. Population genetic groups obtained with K=5.

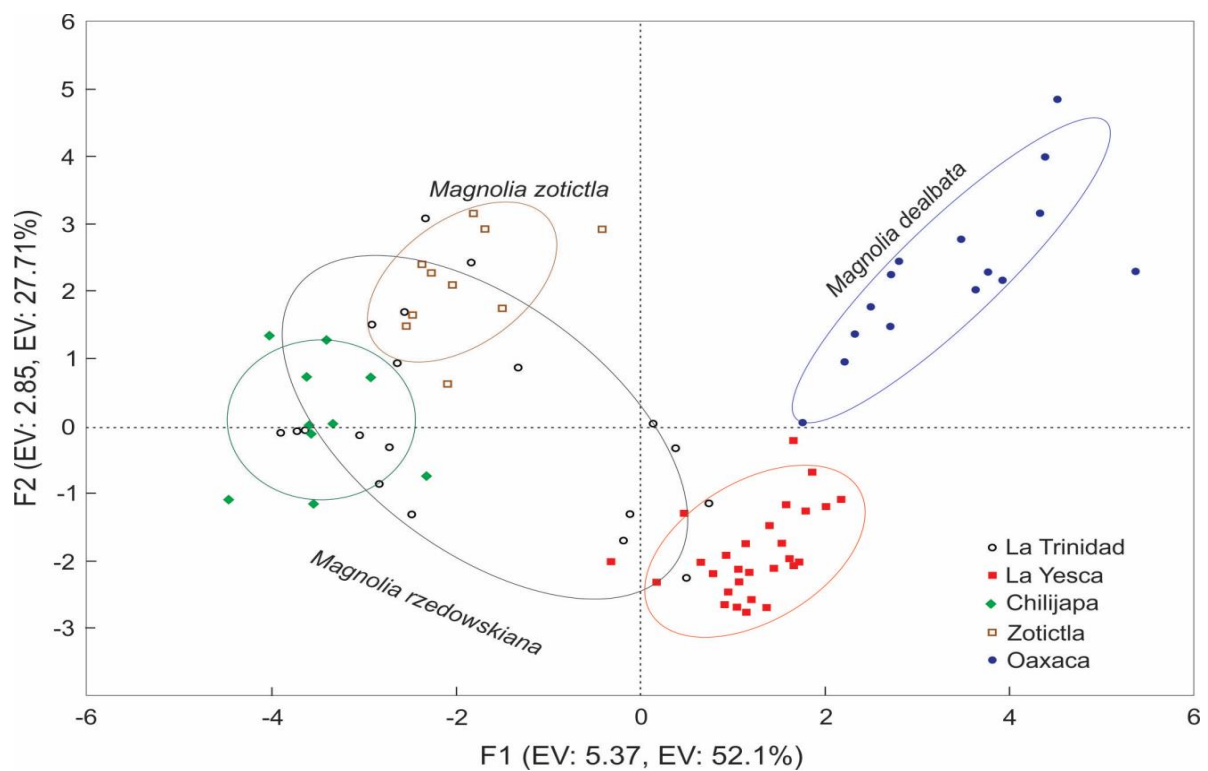


Figure 3

Discriminant function analysis of principal components (DFAPC) of genetic distances of the populations of *Magnolia dealbata*, *M. rzedowskiana*, and *M. zotictla*. F: Discriminant function, EV: Explained variance.

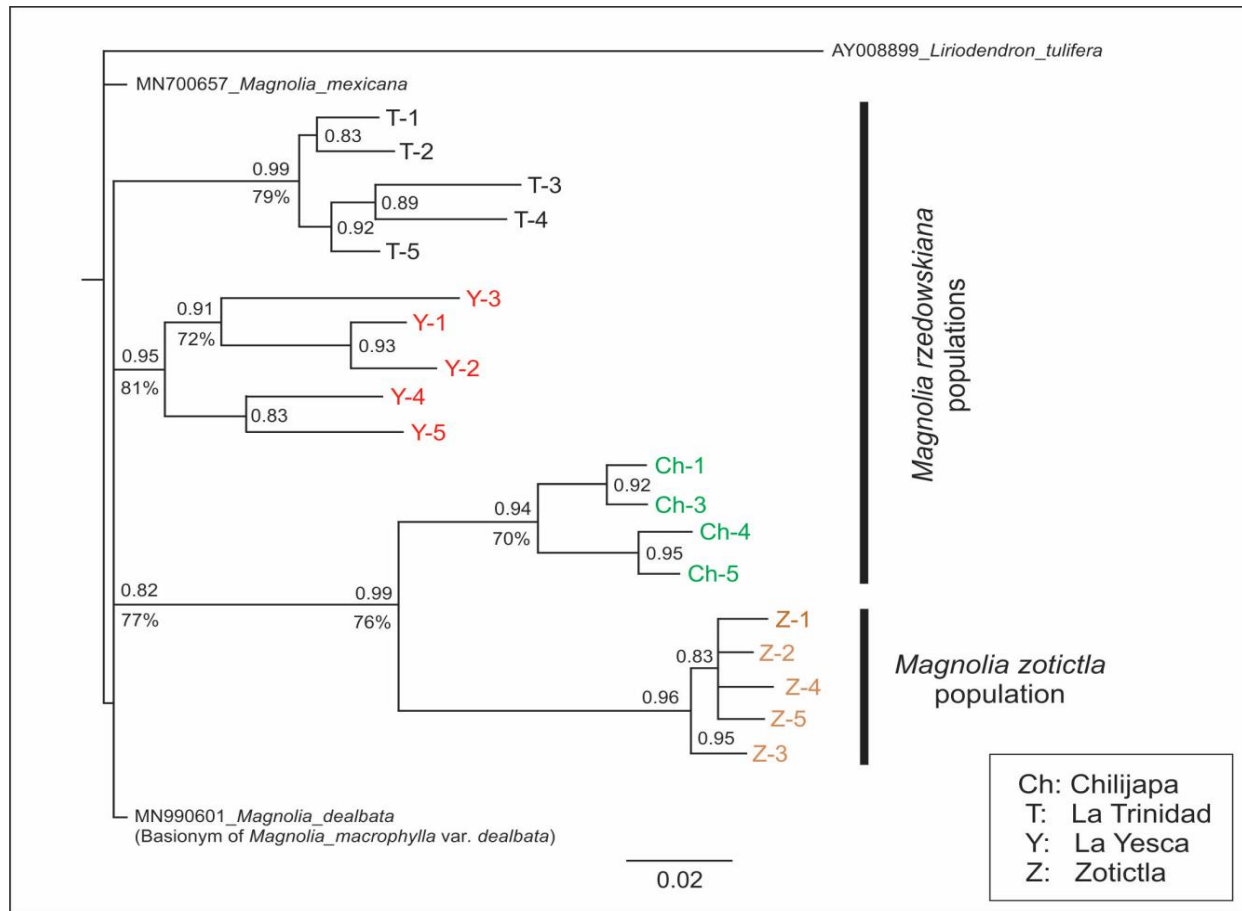


Figure 4  
50 % majority consensus Bayesian phylogenetic tree inferred from sequences of three chloroplast genomic regions (trnH-psbA, ORF350, and rpl32-trnL). Posterior probability values are indicated at the nodes. Bootstrap values  $\geq 70$  % probability are also included.

them (unlike the *M. zotictla* population,  $F_{is} = 0.018$ ), which could be the result of the decrease in gene flow due to the high degree of fragmentation of its habitat (Coates et al., 2018; Wang et al., 2018; Fan et al., 2020). *Magnolia* flowers are pollinated by beetles (Coleoptera), which favors cross-fertilization. However, there is evidence of inbreeding in several species of the genus through geitonogamy, with limited pollen dispersal mechanisms (Chávez-Cortázar et al., 2021; Hernández-Vera et al., 2021). In *M. rzedowskiana*, limited gene flow may be related to geitonogamy and reproduction between related individuals due to low density within isolated populations.

### Genetic structure

Genetic structure is related to the distribution of genetic diversity within and between populations (Wang et al., 2018), and is determined mainly by environmental conditions and by micro-evolutionary processes such as selection, recombination, mutation, and gene flow (Veltjen et al., 2019; Teste et al., 2022). Gene flow and genetic differentiation coefficient are

negatively correlated. Gene flow prevents genetic independence of populations and typically unifies variation (Wang et al., 2020). The value of the differentiation coefficient ( $F_{st} = 0.288$ ) indicates that there is high divergence between the populations of the two species. In addition to this, the low number of migrant individuals per generation ( $N_m = 0.62$ ) confirms that populations behave as independent evolutionary units and indicates that the presence of bottlenecks, genetic drift, inbreeding, and/or limited genetic flow may be the cause of high differentiation (Frankham et al., 2015; Tamaki et al., 2018; Wang et al., 2018). The values of  $F_{st}$  and  $N_m$  support, partially, the taxonomic delimitation of the analyzed species. The low density and geographical isolation between populations, caused by disturbance and fragmentation of the habitat in which they grow (Gutiérrez-Lozano et al., 2020; Sánchez-González et al., 2021), could be related to the strong genetic structure (Fan et al., 2020), except between the populations of La Trinidad (*M. rzedowskiana*) and Zotictla (*M. zotictla*), which have low differentiation values ( $N_m = 2.29$ ,  $F_{st} > 0.10$ ), probably because they share a recent common ancestor.

In the present study, Bayesian analysis divided the populations into five groups or lineages, with different combinations of genetic information. These results were complementary to those obtained from the neighbor-joining classification, and with the DAFPC. The genetic structure found with these analyses is related, in general, to the demographic characteristics of the populations and their taxonomic identity. In the population of *M. rzedowskiana* from La Trinidad, which grows in ideal environmental conditions (little disturbance from human activity) and has a high density of individuals, four of the five genetic groups are present, although only two of them are well represented. This suggests that the La Trinidad population holds more diversity, and coincides with the biogeographic origin of the group (Azuma et al., 2001; García-Morales et al., 2017). The other two populations of *M. rzedowskiana* (Chilijapa and La Yesca) share little genetic information with each other, but each of them is related to the La Trinidad population. As expected, the *M. dealbata* population (outgroup) has unique genetic information, which clearly differentiates it from the other two species of the *Macrophylla* section (Azuma et al., 2001; Chávez-Cortázar et al., 2021).

On the other hand, the simplest interpretation of the genetic relationship between the populations of *M. rzedowskiana* (La Trinidad and Chilijapa) and *M. zotictla* (Zotictla) is that they share a recent common ancestor (Veltjen et al., 2019; Teste et al., 2022); although alternatively it could also be interpreted as a discrepancy caused by the different levels of heterozygosity in the populations examined (Zhao et al., 2012). High genetic differentiation would be expected between these two populations, because the possibility of genetic exchange is very low due to the wide geographical distance that separates them and because they have a wide gap in their flowering periods (Gutiérrez-Lozano et al., 2020; Sánchez-González et al., 2021). In addition, it is common in several *Magnolia* species that pollen and seed dispersal mechanisms are limited, and that the probability of seedling survival is low (Fan et al., 2020; Muñoz-Castro et al., 2020; Chávez-Cortázar et al., 2021; Zhou et al., 2021). It is postulated that allopatric speciation has been the main cause of the diversification of the *Magnolia* genus in the Neotropics (Vázquez-García et al., 2016), so the genetic relationship between *M. rzedowskiana* and *M. zotictla* could be the result of a shared ancestral polymorphism, due to its recent diversification, as suggested by Teste et al. (2022), to explain the genetic structure in some species of the *Talauma* section.

Chávez-Cortázar et al. (2021) evaluated the diversity and genetic structure of six populations of *M. rzedowskiana*. However, their results differ from those obtained in the present study, perhaps because they used different microsatellite markers. For example,  $H_e$  values in the populations they analyzed ranged between 0.10 and 0.70 versus 0.53–0.56 (present study); and  $F_{is}$  values between –0.08 and 0.75, a very wide range of variation within the populations, versus –0.23–0.19 (present study), which indicates greater homogeneity between individuals within populations. Zhao et al. (2012) state that it is common to find contrasting results of this type with different molecular markers because the specificity of each marker is of great relevance in the detection of polymorphisms. In addition,

there are other differences in the analyses carried out in the two studies. Chávez-Cortázar et al. (2021) used leaf samples from only 10 individuals, drawn from small populations (<100 individuals), so the individuals are likely to be closely related. In contrast, in the present study the density was higher; more than 300 trees per population (except Chilijapa, with around 70 trees), and the samples were collected from individuals far apart from each other (at least 50 m) to avoid the effect of inbreeding (Muñoz-Castro et al., 2020; Gutiérrez-Lozano et al., 2023). It is also important to consider the probable presence of null alleles in microsatellite markers used, which can lead to underestimation of genetic diversity values as well as biased interpretations of population structure (Muñoz-Castro et al., 2020).

### Phylogenetic analysis

In the phylogenetic analysis, a pattern of genetic differentiation similar to that obtained through the use of microsatellite markers was observed, in which the populations are each recovered as independent groups or lineages. The populations of Chilijapa (*M. rzedowskiana*) and Zotictla (*M. zotictla*) formed a monophyletic group (PP = 0.99, 76 %); however, within this clade two independent subgroups or lineages were recovered, which correspond to the two species, suggesting that they were genetically connected in the past but that they are currently in the process of speciation along a north–south geographic gradient. *M. zotictla*, being a peripheral population at the extreme south, has become more differentiated from the populations of *M. rzedowskiana* located in the extreme north (Budd et al., 2015). As speciation is typically a process that progresses over long time scales on a population–species continuum (Coates et al., 2018), it is likely that each of the *Magnolia* populations, once it was reproductively isolated, would have been subjected to different selection pressures that were more contrasting at the extremes of their distributions.

Chávez-Cortázar et al. (2021) have suggested using, in addition to microsatellites, other morphological and molecular markers (nuclear and chloroplast genetic sequences), and including samples from populations throughout the species' distribution ranges to elucidate the number of taxonomic units in the *Macrophylla* section. In line with this comprehensive approach, Rico et al. (2021) and Teste et al. (2022) state that to achieve a precise delimitation of the *Magnolia* taxa of the *Magnolia* and *Talauma* sections, it is necessary to achieve a certain degree of understanding of the evolutionary processes involved in their differentiation. This can be achieved through ecological, morphological, and molecular studies in their distribution area, as was done in the present work.

To date, only in *M. rzedowskiana* and *M. zotictla* (species of the *Macrophylla* section that are distributed in Mexico) have quantitative studies been carried out on morphological (Gutiérrez-Lozano et al., 2020) and anatomical variation (Cuapio-Hernández, 2022) at the population level throughout the distribution area. The results of both studies show a significant phenotypic differentiation pattern among all populations (more evident between species), similar to that obtained in the present study at the genetic level. The similarity in the patterns

of genetic, phylogeographic, and phenotypic differentiation (Gutiérrez-Lozano et al., 2020; Cuapio-Hernández, 2022) between the two *Magnolia* species analyzed indicates that there is reproductive isolation and confirms that their delimitation as independent taxonomic units is correct (Coates et al., 2018; Teste et al., 2022).

### Implications for management and conservation

Genetic diversity is generally considered to be the basis of evolutionary change in living organisms; populations that lack genetic variation cannot adapt to changes in environmental conditions and are therefore more susceptible to extinction (Coates et al., 2018; Fan et al., 2020; Zhou et al., 2021; Teste et al., 2022; Chung et al., 2023). A large number of *Magnolia* species that in the past had wide geographical distributions and high population density are now represented by small populations with discontinuous distributions that are reproductively isolated from each other, as is the case of *M. rzedowskiana* and *M. zotictla*, which are therefore considered at risk of extinction (Gutiérrez-Lozano et al., 2020; Chávez Cortázar et al., 2021; Sánchez-González et al., 2021).

Several strategies have been proposed to maintain gene flow between populations among different *Magnolia* species. The first step is to prevent the disappearance of their habitat, and then to reestablish gene flow through artificial pollination or by collecting, dispersing, and cross-sowing seeds between populations (Zhao et al., 2012; Budd et al., 2015; Aldaba Núñez et al., 2021; Fan et al., 2020; Zhou et al., 2021). However, the phenotypic and genotypic differences between the populations within and between the *Magnolia* species analyzed indicate that they behave as independent evolutionary units (with strong adaptation to local environmental conditions), so genetic rescue strategies (artificial dispersion of pollen and seeds) could be inadequate, since they may increase the risk of introducing genes that affect local adaptation and of producing outbreeding depression (Frankham et al., 2015; Aldaba-Núñez et al., 2021; Chung et al., 2023).

The implementation of effective conservation strategies that aim to protect both species and genetic diversity depends largely on how we delimit species or other taxonomic categories (Frankham et al., 2015; Coates et al., 2018; Chávez Cortázar et al., 2021; Teste et al., 2022). The integration of the results on the anatomical, morphological, and genetic variation of the populations of *M. rzedowskiana* and *M. zotictla* are complementary and serve as a guide to set conservation priorities, both of the taxa and of the processes that generated them, considering that each of the populations analyzed has a unique evolutionary history.

The small number of geographically isolated natural populations of *M. rzedowskiana* and *M. zotictla*, as well as their genetic characteristics (intermediate-high diversity, strong genetic structure, little or no gene flow) and high phenotypic plasticity (Gutiérrez-Lozano et al., 2020; Cuapio-Hernández, 2022), are indicators that all populations must be protected (Muñiz-Castro et al., 2020). In *M. rzedowskiana*, the number of individuals in the analyzed populations is relatively high (> 300 adult individuals), so *in situ* management and conservation

strategies can be prioritized (Frankham et al., 2015). Currently, in the towns of Chilijapa and Zotictla, the *ejidatarios* (communal forest owners) are using seedlings obtained by germinating seeds in local nurseries to restore the cloud forest. In addition, the civil authorities of Chilijapa are starting environmental education workshops as a preventive measure to avoid the excessive extraction of *M. rzedowskiana* flowers that the people use in the patron saint festival in May or that they sell in surrounding towns. In any case, it is necessary to generate complementary information on basic demographic aspects of the populations of both species, such as, for example, seed germination rate, seedling viability, generation time, and number of adult plants (Galván-Hernández et al., 2020), among other characteristics, to develop viable management and conservation proposals.

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