Population Genetic Diversity and Structure of Threatened Magnolia Species in Western Mexico

Miguel Angel Muñiz-Castro
Universidad de Guadalajara
Gabriela Lopez-Barrera
Universidad Nacional Autónoma de México: Universidad Nacional Autonoma de Mexico
Maried Ochoa-Zavala
Universidad Nacional Autónoma de México: Universidad Nacional Autonoma de Mexico
Patricia Castro-Felix
Universidad de Guadalajara
Jose Antonio Vazquez-Garcia
Universidad de Guadalajara
Ken Oyama (kenoyama@unam.mx)
Universidad Nacional Autonoma de Mexico https://orcid.org/0000-0002-0367-1964

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Abstract

Background: Genetic diversity is needed to preserve the capability of a species to survive to environmental changes. Due to the presence of small isolated populations, relict species such as *Magnolia* are at an elevated extinction risk. In recent years, many new species of *Magnolia* have been described in Mexico, each one classified by its category of risk. To achieve conservation, knowledge of their basic level of biological diversity is essential to design adequate conservation plans and avoid the negative consequences of genetic loss. Here, we implemented nuclear microsatellite markers to assess 13 populations of three new species of *Magnolia* that were all previously considered to be *Magnolia pacifica*. We aimed to evaluate the genetic agreement with the distinction of these three different morphological species (e.g., their species integrity) and to determine their levels of genetic diversity and their geographic distribution to propose conservation strategies.

Results: We found high levels of genetic diversity compared to other *Magnolia* species with no sign of inbreeding. We found a small effective population size and a prevalence of bottlenecks in some populations. The patterns of genetic subdivision did not support the current morphological distinction of three different species. Instead, we suggest that the genetic structure pattern is the result of historical connectivity and the continuous natural fragmentation of the forest. Thus, an isolation by distance pattern may have had an important role in shaping allele frequencies, producing local genetic differences.

Conclusions: We argue that a major threat underlies the actual trends of habitat loss, which can directly impact the loss of genetic diversity in the current adult individuals and consequently, increase the risk of extinction in further generations. For conservation purposes, we suggest combining *in situ* and *ex situ* conservation of populations with the maintenance of connectivity among the local populations.

Background

A major goal in conservation genetics is the preservation of species as dynamic entities capable of surviving environmental changes, and the maintenance of genetic diversity is a prerequisite to assure this objective [1]. Particularly, relict species are of special concern because they typically have small isolated populations that are prone to the loss of genetic diversity due to random processes and increased levels of inbreeding, leading to an elevated extinction risk [2].

The family Magnoliaceae has an ancient evolutionary history of more than 100 Myr and is considered to be one of the earliest extant lineages of flowering plants [3]. At present, populations of most of the species remain in small patches of temperate and tropical forests of eastern and southeastern Asia and America. Biogeographic studies support the hypothesis that the current distribution of magnolias is the result of several migration events across the Bering and North Atlantic land bridges, and subsequently, their occupied forests became fragmented by geologic and climatic changes [4, 5].

Around two-thirds of the *Magnolia* species are found in Asia; however, in the Neotropics, a large number of new species of *Magnolia* have been described, forming a second center of diversity [6]. The heterogeneous landscape and climate of Mexico support the allopatric speciation of the *Magnolia* genus [7, 8, 9]. In western Mexico, along the Mexican Pacific slopes, *Magnolia pacifica* comprised three subspecies restricted to the Sierra Madre Occidental (west of Mexico) and the westernmost region of the Trans Mexican Volcanic Belt (center of Mexico) and Sierra Madre del Sur (south of Mexico): *M. pacifica* subsp. *pacifica*, *M. pacifica* subsp. *tarahumara* and *M.*

pacifica subsp. *pugana* [7]. A major morphological revision allowed for the recognition of *M. pacifica* subsp. *pugana* as separate taxon called *M. pugana* [10]. Additionally, three populations geographically and morphologically close to *M. pacifica* were identified as *M. vallartensis* [11]. These related species occupy different habitats along a moisture gradient [12]; *M. pacifica* is distributed in cloud forests throughout the Pacific slope from Nayarit to Jalisco states, between 750 and 1900 m in altitude. *Magnolia pugana* is a more continental species distributed from central Jalisco to Zacatecas states, between 1300 and 1800 m, occurring on steep slopes or ravines with forested margins along with a permanent water supply (streams) [7, 10]. In contrast, *M. vallartensis* can be found in the transition zones between cloud forests and pine-oak forests and subtropical sub-evergreen forests at a lower elevation than *M. pacifica* and *M. pugana*, from 100 to 1000 m [11].

Magnolia species are valued for their wood, food, floral scents, medicinal properties and ornamental uses [13] but are also of great scientific interest due to their evolutionary and biogeographic history [14, 15, 16]. The Magnolia family consists of approximately 350 species, with a large number of these species (*ca.* 130) threatened with extinction in the wild [6]. In this sense, *M. pacifica* and *M. pugana* are classified as endangered, while *M. vallartensis* is critically endangered [6]. Current threats to *Magnolia* species are severe habitat reduction, overharvesting, poor fruiting, reduction of pollinators, seed predation and a low rate of natural regeneration [6, 15, 17, 18]. The natural populations of *Magnolia* present a disjunctive distribution and tend to be scanty, whereby habitat loss and fragmentation, as well as overharvesting, make them highly vulnerable [6, 15, 16]. Thus, knowledge of the basic level of biological diversity is essential to design adequate conservation plans and to avoid the negative consequences of genetic loss.

Population genetic studies in the Americas showed different levels of genetic diversity and gene flow patterns, suggesting that population parameters are highly dependent on the strength of stochastic forces that each *Magnolia* species experienced. North American populations of *M. acuminata* and *M. schiedeana* have high genetic diversity with moderate to high genetic structure [19, 20], and gene flow between populations of *M. cubensis* could be facilitated because their seeds can be transported among fragmented habitats by birds [21, 22]. Even within the same geographic region, different patterns of gene flow were suggested for *Magnolia* species in southern [23] and western [12] Mexico. Because of their rarity and the continuous decline of the habitat of magnolias, and the need for genetic information to guide management decisions in Mexico, we implemented nuclear microsatellites markers to assess 13 populations of three closely related species of *Magnolia*: *M. pacifica*, *M. pugana*, and *M. vallartensis*. We aimed to: 1) determine the level of intrapopulation gene diversity and how it is geographically distributed; 2) evaluate the genetic agreement with the distinction of these three different morphological species (e.g., species integrity); and 3) propose appropriate conservation strategies.

Results

Data checking

Overall, we did not detect genotyping errors or the presence of null alleles across loci. Only localities from Vallarta 2 (LAJ), Arroyo Virgen (AVI), and El Encanto (ENC) showed one locus (Stm0200) with a null allele frequency of 0.150, 0.219 and 0.362, respectively. The exact test of Hardy-Weinberg indicated deviations from equilibrium in eight of 13 localities (Table S1), as well as in all loci across localities (Table S2). We did not detect an association between pairs of loci across populations (Table S3).

Species integrity and genetic structure

We detected a positive correlation between genetic and geographical distances (r = 0.326; p = 0.0009) that represents geographical restricted dispersal between populations (IBD). Contrary to what we expected, both approaches failed to group populations according to three different species (Fig. S1). Inspection of the ΔK plot from STRUCTURE analysis indicated two major clusters that showed shared ancestry (Fig. 2). The former merged all populations of *M. vallartensis* and *M. pacifica* (west cluster). On the other hand, all populations of *M. pugana* (east cluster) formed the second cluster (Fig. 3b). Although ΔK suggested support for K= 2, it also showed a clear secondary peak at K= 9 (Fig. 2), suggesting other levels of organization present in the population structure.

The plot of the cross-validation score from TESS 3 did not exhibit an explicit plateau; however, we observed that the plateau started at K= 6 (Fig. 2). Similar to the Bayesian approach, this suggested a much finer population structure, possibly because IBD has a role in shaping allele frequencies. The six inferred genetic groups were geographically coherent (Fig. 3a); in general, nearby populations were assigned to the same cluster. However, there were also close populations assigned to different clusters (Fig. 3b). Populations from *M. vallartensis* (PMA, LAJ, and CCO) formed a genetic group. The northern and southernmost populations of *M. pacifica* (SMP and TAL, respectively) formed two separate clusters, while two populations of *M. pacifica* and one of *M. pugana* formed another genetic group [San Juan (SJU), San Sebastián (SSE), and Río Verde (RVE)]. To the east of the *Magnolia* distribution, Palo Verde (PVE), Arroyo Virgen (AVI), and El Encanto (ENC) were clustered together, and Arroyo Sampurrón (AS) and San José (ASJ) were assigned to another genetic group (Fig. 3a, b).

The AMOVA results indicated that the highest percentage of variation was within individuals (87 %) for both grouping hypotheses (Table 3). Only 2.37 % and 10.26 % of the genetic variation occurred between the two (F_{CT}= 0.024; p = 0.140) and six (F_{CT}= 0.102; p = 0.003) genetic groups detected for STRUCTURE and TESS 3, respectively.

We obtained substantially higher values of D than G_{ST} (Fig. 4). The mean pairwise statistics of D (0.616) and G_{ST} (0.103) indicated that the populations share approximately 40 % of their alleles at relatively low frequencies. D ranged from 0.075 to 0.866 and G_{ST} from 0.021 to 0.153; see Table S4 for the lower and upper confidence intervals. Regarding the D values, we observed high levels of allelic differentiation among most populations, and considerably higher allelic differentiation between the west and east clusters (Fig. 4). We identified some population pairs with higher values of G_{ST}, which involved mainly populations from the east; however, these populations were still far from fixation (Fig. 4).

The NJ tree showed three major lineages that did not correspond to different species (Fig. 5). Excepting the populations from Vallarta 2 (LAJ) and Río Verde (RVE), the NJ result seems to be consistent with K= 2; however, it also shows some inconsistencies with the geographic distribution of the populations (Fig. 5). Despite this, the NJ showed a close relationship among some populations at the east end of the *Magnolia* distribution (ENC, AVI, and PVE) and that Cabo Corrientes (CCO) and Santa María (SMP) shared common ancestry (Fig. 5).

Genetic diversity and effective population size

We obtained a total of 157 alleles with 26.167 ± 6.432 (mean ± SD) alleles across all microsatellite loci. At the species level, we observed high levels of heterozygosity, which ranged from 0.638 to 0.729 for Ho and from 0.688

to 0.773 for He (Table 4). However, *M. pugana* showed the lowest allelic richness (9.959) compared to *M. vallartensis* (11.980) and *M. pacifica* (14.160). In particular, we observed overall a decrease of allelic richness towards the eastern cluster (*M. pugana*) in contrast to the western ($p < 0.05$). The inbreeding coefficient (F_{IS}) ranged from −0.073 to 0.209, but only *M. pacifica* showed a p-value of 0.004 (Table 4). Across populations, we observed overall high levels of Ho and He, which ranged from 0.487 to 0.786 and from 0.668 to 0.842, respectively (Table 4). The allelic richness (Ar) showed values from 4.407 to 9.27, which revealed three localities of *M. pugana* with the lowest values of Ar: Arroyo Sampurrón (4.407), Arroyo San José (4.950), and El Encanto (4.860). The last one also showed lower values of Ho and He (0.487 and 0.6704, respectively). The inbreeding coefficient showed five populations with positive values and eight indicating an excess of heterozygotes; however, none had a probability lower than 0.05 to reject the null hypothesis (Table 4).

We estimated the effective population size (*Ne*) based on the six genetic clusters identified previously by TESS 3. Most populations had lower values of *Ne*, which ranged from seven to 483 individuals (Table 5). The genetic cluster with the lowest *Ne* corresponded to localities from Arroyo Sampurrón (AS) and Arroyo San José, and the cluster with the highest value of *Ne* corresponded to Santa María (SMP). On the other hand, regarding genetic bottlenecks, genotype data showed a tendency of deviation from mutation-drift equilibrium in populations located in the east (from PVE to RVE; Table 6).

Discussion

Species integrity and genetic structure

Species delimitation is of critical importance in conservation biology because the numbers of species reflect attributes that can be compared across taxa and localities, such as threats, richness, endemism, and diversity [52, 53]. Species delimitation based on morphological traits alone can mask the presence of cryptic species or overestimate the diversification of a single species [54]. The integration of several approaches (e.g., species distribution, molecular data and morphological traits) improves our understanding of the patterns of speciation [55].

In *Magnolia*, some populations of *M. pacifica* were originally proposed as separate subspecies, which subsequently were raised to full species level [7, 10, 11]. According to this, the number of carpels per fruit and the number of stamens per flower distinguished *M. pugana* from *M. pacifica* [10]. While *M. vallartensis* differed from *M. pacifica* by the presence of a colorful abaxial base of outer petals, leaf shape and size, fewer carpels and more petals number [11]. Here, we did not find good accordance with this morphological distinction that has motivated the designation of three different species. This was supported by clustering results, the neighbor-joining tree and measures of allelic differentiation. Thus, we hypothesized that these populations are characterized by a combination of genetic clusters and local differentiation due to geographic restrictions on gene flow, which reflect the different levels of organization present in the genetic structure. However, a great proportion of the genetic variation affecting phenotypic adaptive traits cannot be detected with neutral markers, and it is well known that for plants in general, the diversity at phenotypic level is larger than at neutral marker level [56].

Different demographic and historical processes may have led to the observed pattern. Clustering results suggested two values of genetic partition: $K = 2$ and $K = 6$ for STRUCTURE and TESS, respectively. Although these

genetic clusters explain a very small fraction of the genetic variation, and *K*= 2 had no statistical support, we observed, however, that for *K*= 2, D values also recovered a higher allelic differentiation among populations of these two clusters, suggesting a break in allele frequencies. The resulted genetic subdivision (*K* = 6) may also suggest a higher local differentiation within the *Magnolia* species complex by the interaction between the response of plants to local climates and the effects of forest fragmentation in the past.

The Mexican cloud forests are the remnants of temperate woody elements that migrated south from northern North America during the early Oligocene [4, 5, 14]. Studies have suggested that since their establishment, mountain regions have undergone repetitive latitudinal and altitudinal migration, promoting the expansion, contraction, and divergence of populations [57]. There are two hypotheses about the role that the Last Glacial Maximum played in the Neotropical cloud forests: the dry refugee scenario, in which species distributions were shifted by aridity (contraction and extinction) and the moist forest hypothesis, in which no significant decrease in precipitation favored downslope migration and population connectivity, while during warm interglacial periods, fragmentation can result in the development of isolation by distance pattern [58].

The inferred gene flow among groups of populations, in particular between two geographically distant populations (SMP and CCO) for which a close relationship was also observed by NJ, and the presence of genetic intermediates between two populations from the west (SJU and SSE) and one population (RVE) from the east cluster (Fig. 3), might support the population connectivity hypothesis. In this context, the presence of isolation by distance and the overall high allelic differentiation obtained among populations, due to limited pollen and seed dispersal under a fragmented landscape, provide additional support for this scenario. The isolation by distance pattern has been found in both temperate and tropical American magnolias [20, 21]. Thereby, we suggest that climatic changes in the recent past impacted forest distribution, leading to fragmentation of the forest in which Mexican *Magnolia* presently occur [23], and thus explaining the geographic distribution of the genetic variation. The influence of the past glacial cycles was also observed in the Japanese *Magnolia kobus* [59] and the relict species *Platanus orientalis*, whose distribution is strongly influenced by humidity and water availability, similar to magnolias [60].

High genetic diversity and no evidence of inbreeding

Population genetic studies of *Magnolia* species have shown different patterns of genetic diversity and gene flow [19, 20, 22, 23], suggesting that the genetic patterns are dependent on the evolutionary history of populations and the strength of stochastic forces that each *Magnolia* species had experienced. We found levels of genetic diversity comparable to those obtained for *M. acuminata*, a species distributed in Canada and the United States, and other species distributed in Japan, such as *M. stellata* and *M. kobus*. The high levels of genetic diversity found in these species were partly explained by historical dynamics [19, 59, 61].

In this sense, the moist forest scenario (discuss above) is consistent with the preservation of genetic diversity through historical gene flow between populations [58]. In addition, the long generation times of trees could buffer the loss of genetic variation within populations, contributing to the maintenance of levels of genetic diversity [19]. On the other hand, the lower allelic richness towards the eastern cluster may be explained by the conjunction of a low effective population size and the prevalence of genetic bottlenecks detected in these populations. If this hypothesis is correct, an enhanced effect of genetic drift in this geographic area may remove alleles within populations and directly disturb allele frequencies, resulting in a decrease of allelic richness eastward and reinforce genetic differentiation. Contrary to what can be expected in small isolated populations, no inbreeding

was detected for the studied populations. This can be explained by the protogynous flower of *Magnolia* and the active movements of its pollinators [62, 63]. Nevertheless, taking into consideration the continuous natural forest fragmentation since glacial periods, and the self-compatibility observed in *Magnolia* species [63], it is also likely that inbreeding remains undetected because insufficient time has elapsed [65].

Conservation of Mexican *Magnolia*

It is well known that genetic variability is needed for the long-term viability of populations and species. In this study, we detected high levels of genetic diversity and high allelic differentiation with alleles at relatively low frequencies. Thus, in order to conserve the whole variation, we need to preserve most of the populations throughout the geographic distribution, which can only be achieved by the establishment of federal protection of these forests. At this point, integrating both *in situ* and *ex situ* collections are pivotal in the preservation of *Magnolia* species [15]. *Ex situ* collections of studied populations have been started by creating seed banks that subsequently may serve as a source of material for the reintroduction and/or reforestation of the same population of origin (*in situ* source population). *Ex situ* collections have been demonstrated to be essential to conservation strategies because they can protect an important fraction of the genetic variation, such as in *Magnolia officinalis subsp. biloba*, among which cultivated populations retain 95 % of the genetic variation of wild populations [65], or in the case of *Magnolia vovidesii* (formerly *M. dealbata*), in which *ex situ* collections have served for reintroduction, propagation and *insitu* restoration programs in Mexico [66].

At the moment, we did not detect any imminent genetic risks in the *Magnolia* trees studied in terms of the levels of genetic diversity and inbreeding coefficients. However, given the endangered status of *Magnolia* species in Mexico, their small effective population size, their scarcity and fragmented habitats, we warn about the negative effects that the persistent anthropic fragmentation may have in further generations. Rates of deforestation are increasing in Mexico [67], producing fragmented forests and isolated natural populations of trees that increase the probabilities of inbreeding and genetic drift that leads to inbreeding depression, genetic degradation and changes in the genetic structure [68, 69].

Conclusions

The results reported here pointed out that the genetic subdivision among populations of the *Magnolia* species is better explained by historical gene flow with isolation by distance. Thus, we suggest that the studied populations represent part of a single species complex that has undergone high genetic differentiation. We can invoke other factors, such as historical connectivity and continuous natural fragmentation, to explain this pattern. Moreover, the high genetic diversity—but not necessarily the same alleles across populations—provide additional support for this hypothesis. Our results also showed a lack of inbreeding across populations with a low effective population size; thus, we argue that a major threat underlies the actual trends of habitat loss, which can directly impact future generations and consequently increase the risk of extinction. Thus, we recommend that *in situ* and *ex situ* conservation actions should be accompanied by implementing strategies focused on the maintenance of connectivity among populations through gene flow, preventing the negative consequences of habitat fragmentation.

Methods

Study sites and species identification

We collected foliar tissue from 160 adult trees through 13 localities (four for *M. pacifica*, six for *M. pugana* and three for *M. vallartensis*) distributed in three states of Mexico (Jalisco, Zacatecas, and Nayarit) (Table 1; Figure 1). All leaf samples were collected from the wild under the permissions authorized by Mexican authorities (SEMARNAT) to MAM-C, PC-F and JAG-V. The localities were selected to geographically represent the entire range of species distribution. There was a minimum distance of 100 m between sampled individuals. Leaves were collected and stored in plastic bags filled with silica gel. Species identification was accomplished through visual examination and measurements of morphological characters determined in the field and the IBUG herbarium (Herbario Luz María Villareal de Puga Instituto de Botánica, Universidad de Guadalajara, México) through voucher specimens collected for reference [7, 10, 11] (Supplementary material, Appendix 1). All voucher specimens of the samples collected for this study are deposited at IBUG herbarium.

DNA extraction and microsatellites amplification

Genomic DNA extraction was performed following the CTAB-based method [24]. The isolated DNA was diluted with deionized water to a final concentration of 25 ng/μL and stored at −20 °C. Samples of *Magnolia* from all three species were amplified using six previously developed primers: M6D1, M10D8 [25], stm0200, stm0218, stm0246 and stm0346 [26] (Table 2). PCRs were performed individually in a total volume of 5 μL containing 2× of QIAGEN Multiplex PCR Kit, 10 pmol of each primer and 25 ng of genomic DNA. PCR was performed in an Eppendorf Mastercycler Nexus with the following conditions: an initial denaturation step of 95 ºC for 15 minutes, followed by 35 cycles of 94 °C for 30 seconds, the annealing temperature for 90 seconds (Table 2), 72 °C for 7 minutes, and a final extension step at 72 °C for 7 minutes. PCR products were combined with a GeneScan-600 LIZ size standard for capillary electrophoresis with an ABI-PRISM 3100 Avant sequencer (Applied Biosystems). Fragments sizes were recorded using the program GenMarker version 3.0.1 (Softgenetics).

Data checking

We tested for the presence of null alleles, large-allele dropout, and errors due to stuttering in the microsatellite data using MICROCHECKER version 2.2.3 [27] with 10000 bootstrap and 95 % confidence interval. Each sample at each locus was tested for conformance to Hardy-Weinberg equilibrium (HWE) and linkage disequilibrium between pairs of loci by each locality. For both analyses, we used GENEPOP on the web version 4.7 [28, 29] using 1000 dememorization numbers, 200 batches, and 1000 iterations per batch.

The integrity of species and genetic structure

To assess the distribution of genetic variation, we first performed a Mantel test to evaluate if populations across the whole range of the three species of *Magnolia* had adjusted to an isolation by distance (IBD) pattern. To do this, we used paired matrices of genetic (FST/(1-FST)) and geographic (km) distances using the program ALLELES IN SPACE [30] version 1.0 with 1000 permutations. Then, to assess the integrity of the species, we probabilistically assigned individuals to distinct genetic groups (*K*) based on multilocus genotypes; we expected to find groups of populations according to different species at $K = 3$. To do so, we implemented two approaches: a Bayesian clustering method with STRUCTURE software version 2.3.4 [31] and the least-squares optimization algorithm of TESS 3, which does not assume linkage or HWE [32].

Overall, for each method, we performed 20 independent runs for each *K* value, which ranged from 1 to 15. For the STRUCTURE program, we assumed the admixture model with correlated allele frequencies. We performed 1000000 of Markov chain Monte Carlo repetitions and 100000 as the burn-in period. To identify the uppermost hierarchical level of the genetic partition, we implemented the Δ*K* method [33] in STRUCTURE HARVESTER [34]. Then, we used CLUMPAK [35] to summarize the results and generate figures for each *K* value.

For TESS 3, we implemented the package 'tess3r' [32] for R software [36]. The algorithm combines matrix factorization and spatial statistical methods to provide estimates of ancestry coefficients [32]. For each *K* value, only those repetitions with the lowest root mean squared errors were retained. We determined the optimal number of ancestral populations based on a cross-validation method. The best choice of *K* usually corresponds to a plateau in the cross-entropy plot [32]. Lower values of the cross-validation score indicate better models in the sense that they are parsimonious and explain the data [37].

To determine the partitions of genetic variation within and among groups, we conducted an analysis of molecular variance (AMOVA) using ARLEQUIN version 3.5 [38]. This analysis was performed based on the stepwise mutation model (SMM), grouping populations according to both clustering outputs. Statistical support was computed using 10000 permutations.

To evaluate the phylogenetic relationships among the populations of closely related species, we constructed a neighbor-joining (NJ) tree based on D_A genetic distance [39] as implemented in POPTREE2 [40]. We evaluated the strength of phylogenetic inferences performing 100000 bootstraps replications.

To quantify complementary aspects of genetic structure [41], we measure allelic differentiation and fixation index by computing pairwise Jost' *D* [42] and G_{ST} [43], respectively. This was implemented in diverRsity package with 100 bootstraps [44].

Genetic diversity, effective population size and bottlenecks

For genetic diversity estimates, we computed the following parameters for each species and locality: the mean number of alleles (Na), the number of effective alleles (Ne), the observed heterozygosity (Ho), the expected heterozygosity (He), and total allelic richness (Ar). We computed Na and Ne using the software GENALEX version 6.501 [45, 46]; Ho and He were estimated using GENETIX version 4.05 [47] and Ar was computed based on a sample size of 20 and 40 alleles (at the population and species level, respectively) using the rarefaction method in the 'hierfstat' package [48]. The inbreeding coefficients (F_{IS}) were computed by ARLEQUIN with 10000 permutations.

Because the effective population size (*Ne*) is one of the most critical parameters in population genetics and conservation biology, we estimated *Ne* with NeEstimator software version 2.1 [49]. We used the amount of linkage disequilibrium [50] and a minimum allele frequency of 0.05. To avoid bias due to gene flow or substructure, we computed estimates of contemporary *Ne* for each genetic cluster defined previously. Confidence intervals (CIs) for *Ne* were obtained from parametric CI.

To determine if reductions in population size may impact the genetic diversity estimates, we performed a sign and Wilcoxon test (two-tail for excess or deficit of heterozygotes) to assess recent changes in population size

using BOTTLENECK version 1.2.02 [51]. To do this, we used 1000 iterations implementing stepwise (SMM) and two-phase (TPM) mutation models with the default parameters.

Abbreviations

AMOVA: Analysis of Molecular Variance; Ar: Allelic richness; CBTA: Cetyltrimethylammonium bromide; CI: Confidence intervals; D_A: Genetic distance; DNA: Deoxyribonucleic acid; F_{IS}: inbreeding coefficient; F_{ST}: Fixation index of the effect of subpopulations compared to the total population; G_{ST}: Total genetic differentiation; He: Expected heterozygosity; Ho: Observed heterozygosity, HWE: Hardy-Weinberg equilibrium; IBD: Isolation by distance; IBUG herbarium: Herbario Luz María Villareal de Puga Instituto de Botánica, Universidad de Guadalajara, México; K: Distinct genetic groups; Na: Mean number of alleles; Ne: Number of effective alleles; NJ: Neighbor-joining; PCR: Polymerase chain reaction; SMM: Stepwise mutation model; TMP: Two-phase mutation model.

Declarations

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Authors' contributions

Conceptualization: KO, MAM-C; Field collection: MAM-C, JAV-G, PC; Laboratory analyses: GL-B, PC; Statistical analysis: MO-Z, GL-B; Writing: KO, MO-Z, GL-B, MAM-C, PC, JAV-G; Final edition: KO, MO-Z. All authors have read and approved the manuscript.

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Availability of data and materials

All data generated or analysed in this study are included in this manuscript and its supplementary information files.

Ethics approval and consent to participate

This research does not involve human subjects. All biological samples were obtained non-invasively in accordance with collection permits from SEMARNAT.

Consent for publication

This manuscript does not relate to an individual person. All authors have read and approved the publication of this manuscript.

Competing interest

The authors declare that they are no competing interests.

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Tables

Table 1. Sample size and geographic coordinates of 13 localities of three species of *Magnolia* from western Mexico.

Species	Population name	Population ID	Sample size	Latitude (N)	Longitude (W)
M. vallartensis	Vallarta (Palo María)	PMA	12	20.5359333	-105.24415
	Vallarta 2 (Lajitas)	LAJ	15	20.4708333	-105.2566333
	Cabo Corrientes	CCO	13	20.3604	-105.2577167
M. pacifica	Santa María	SMP	14	22.7026883	-105.1821615
	San Juan	SJU	12	21.46585	-105.00575
	San Sebastian	SSE	12	20.7560167	-104.8419333
	Talpa	TAL	12	20.2320333	-104.7709
M. pugana	Palo Verde	PVE	12	21.2610667	-103.3054
	Arroyo Sampurron	AS	11	21.0571667	-103.2395333
	Arroyo San José	ASJ	11	21.030817	-103.25295
	Arroyo Virgen	AVI	12	20.8159833	-103.5802667
	El Encanto	ENC	12	20.8036	-103.5563
	Río Verde	RVE	12	20.7343167	-103.1841667

Table 2. Nuclear microsatellite markers used to genotype three *Magnolia* species from western Mexico.

Locus	Repeat motif	Sequence 5'-3'	Ta (º C)			Reference
			Magnolia vallartensis	*Magnolia pacifica*	*Magnolia pugana*	
M6D1	$(CT)_{43}$	F: ACT GGA GCA GTG CCT GGA TA R: TCG CAA CTG CGT GTT CTC AT	60.1			Isagi *et al*., 1999
M10D8	$(GAA)_6$ $(GA)_{26}$	F: AGC CCT CTA TAC ACG CAC ACA T R: CGG AGC TAC AAG GAG CAG AAT A	60.1			Isagi *et al*., 1999
stm0200	$(CT)_{13}$ $(TC)_{11}$	F: GCAAGCTACCAGGTTACTC R: AGCCATCTGATGTTTTGATAC	59.5		60.1	Setsuko *et al*., 2005
stm0218	$(ACC)_4$ $(AG)_{28}$	F: CCACCACCACTGCCGAATCT R: TCGCCTCAAAATGTCATTGGA	60.1			Setsuko *et al*., 2005
stm0246	$(GA)_{33}$	F: AAGCAAAGCCTCCTAGGTC R: TCTACGCCTAACAGGTCTGTC	60.1			Setsuko *et al*., 2005
stm0349	$(GA)_{14}$ $(GA)_8$	F: TTGTCCATGAAGTGTGTAAA R: GATCCAGGATTATTACCAGTC	53.8			Setsuko *et al*., 2005

Table 3. Results of analysis of molecular variance (AMOVA) of clustering individuals of three species of *Magnolia*.

Source of variation	Percent of variation		F-statistics				
	K= 2	*K*= 6		*K*= 2		*K*= 6	
Among groups	2.37	10.26	F_{CT}	0.024	(0.14)	0.102	(0.003)
Among populations within groups	13.88	6.18	F_{SC}	0.140	(0)	0.069	(0)
Among individuals within populations	-4.07	-4.07	F_{IS}	-0.049	(0.827)	-0.049	(0.82)
Within individuals	87.82	87.64	F_{IT}	0.122	(0.014)	0.124	(0.014)

We used the stepwise mutation model; p-values from *F*-statistics were included within parentheses.

Table 4. Estimates of genetic diversity of 13 populations of three *Magnolia* species from western Mexico.

Species	Population	ID	Na[a]	Ne[a]	Ar	Ho[b]	He[b]	F_{IS}	p-value
M. vallartensis	Vallarta (Palo María)	PMA	6.667 (0.882)	4.310 (0.747)	6.434	0.683 (0.2057)	0.702 (0.2248)	0.0337	0.435
	Vallarta 2 (Lajitas)	LAJ	6.833 (0.792)	4.463 (0.740)	6.912	0.628 (0.143)	0.729 (0.1291)	0.078	0.316
	Cabo Corrientes	CCO	6.833 (0.946)	4.867 (0.796)	6.191	0.712 (0.1576)	0.746 (0.1292)	-0.159	0.815
	TOTAL		6.778 (0.475)	4.547 (0.417)	11.980	0.638 (0.038)	0.688 (0.038)	0.001	0.495
M. pacifica	Santa María	SMP	6.667 (0.919)	3.707 (0.838)	5.441	0.619 (0.121)	0.678 (0.1251)	0.097	0.294
	San Juan	SJU	9.333 (0.760)	6.800 (0.708)	9.277	0.786 (0.0500)	0.842 (0.1219)	-0.235	0.902
	San Sebastian	SSE	9.333 (0.760)	5.720 (0.713)	8.349	0.736 (0.0549)	0.811 (0.0974)	0.201	0.103
	Talpa	TAL	6.333 (0.494)	4.377 (0.432)	6.133	0.775 (0.049)	0.761 (0.118)	0.216	0.111
	TOTAL		7.917 (0.458)	5.151 (0.407)	14.160	0.729 (0.026)	0.773 (0.019)	0.209	0.004
M. pugana	Palo Verde	PVE	5.667 (0.667)	3.919 (0.627)	5.414	0.622 (0.1251)	0.7056 (0.1977)	-0.424	0.996
	Arroyo Sampurron	AS	4.833 (0.601)	3.371 (0.507)	4.407	0.754 (0.1187)	0.6681 (0.1083)	-0.456	0.984
	Arroyo San Jóse	ASJ	4.833 (0.872)	3.350 (0.401)	4.950	0.757 (0.1086)	0.6756 (0.1369)	-0.197	0.794
	Arroyo Virgen	AVI	5.333 (0.715)	3.543 (0.393)	5.197	0.597 (0.0815)	0.6999 (0.1833)	-0.02	0.544
	El Encanto	ENC	5.667 (0.803)	3.710 (0.632)	4.860	0.487 (0.1931)	0.6704 (0.2535)	-0.188	0.853
	Río Verde	RVE	6.667 (0.715)	3.907 (0.611)	6.184	0.611 (0.1191)	0.7082 (0.1884)	-0.282	0.932
	TOTAL		5.500 (0.297)	3.633 (0.207)	9.959	0.675 (0.033)	0.726 (0.020)	-0.073	0.845

Na: the mean number of alleles; Ne: the effective number of alleles; Ar: allelic richness; Ho and He: the observed and expected heterozygosity, respectively; F_{IS}: inbreeding coefficient; p-value of F_{IS}. [a] mean (± standard error); [b] mean (± standard deviation).

Table 5. Estimates of effective population size (*Ne*) for each genetic cluster identify for TESS 3.

Genetic cluster	Population (ID)	*Ne*	95 % CI lower	95% CI upper
1	Vallarta (PMA)	15.4	11.4	21.0
	Vallarta 2 (LAJ)			
	Cabo Corrientes (CCO)			
2	Santa María (SMP)	483.0	18.5	Infinite
3	San Juan (SJU)	22.5	15.8	33.9
	San Sebastián (SSE)			
	Río Verde (RVE)			
4	Talpa (TAL)	53.2	9.1	Infinite
5	Palo Verde (PVE)	55.8	26.4	338.4
	Arroyo de la Virgen (AVI)			
	El Encanto (ENC)			
6	Arroyo Sampurrón (AS)	7.5	3.4	13.1
	Arroyo San José (ASJ)			

Table 6. Test for null hypothesis under the stepwise (SMM) and two-phase (TPM) mutation models. Bold values $p < 0.05$.

	Sign test		Wilcoxon test	
Population name (ID)	**TPM**	**SMM**	**TPM**	**SMM**
Vallarta (PMA)	**0.037603**	0.190257	**0.015625**	0.109375
Vallarta 2 (LAJ)	0.227106	0.461279	0.109375	0.84375
Cabo Corrientes (CCO)	0.466411	0.494155	0.109375	0.84375
Santa María (SMP)	0.139887	0.467137	**0.046875**	0.5625
San Juan (SJU)	0.463354	0.45447	1	0.4375
San Sebastian (SSE)	0.537235	0.193033	0.4375	0.4375
Talpa (TAL)	0.211004	0.213411	**0.03125**	0.078125
Palo Verde (PVE)	**0.032946**	**0.027634**	**0.015625**	**0.015625**
Arroyo Sampurron (AS)	0.175884	0.200122	**0.03125**	**0.046875**
Arroyo San José (ASJ)	**0.031232**	**0.040356**	**0.015625**	**0.015625**
Arroyo Virgen (AVI)	0.510246	0.458935	0.078125	0.109375
El Encanto (ENC)	**0.040522**	0.201481	**0.015625**	**0.046875**
Río Verde (RVE)	0.482094	0.540249	0.078125	0.84375

Figures

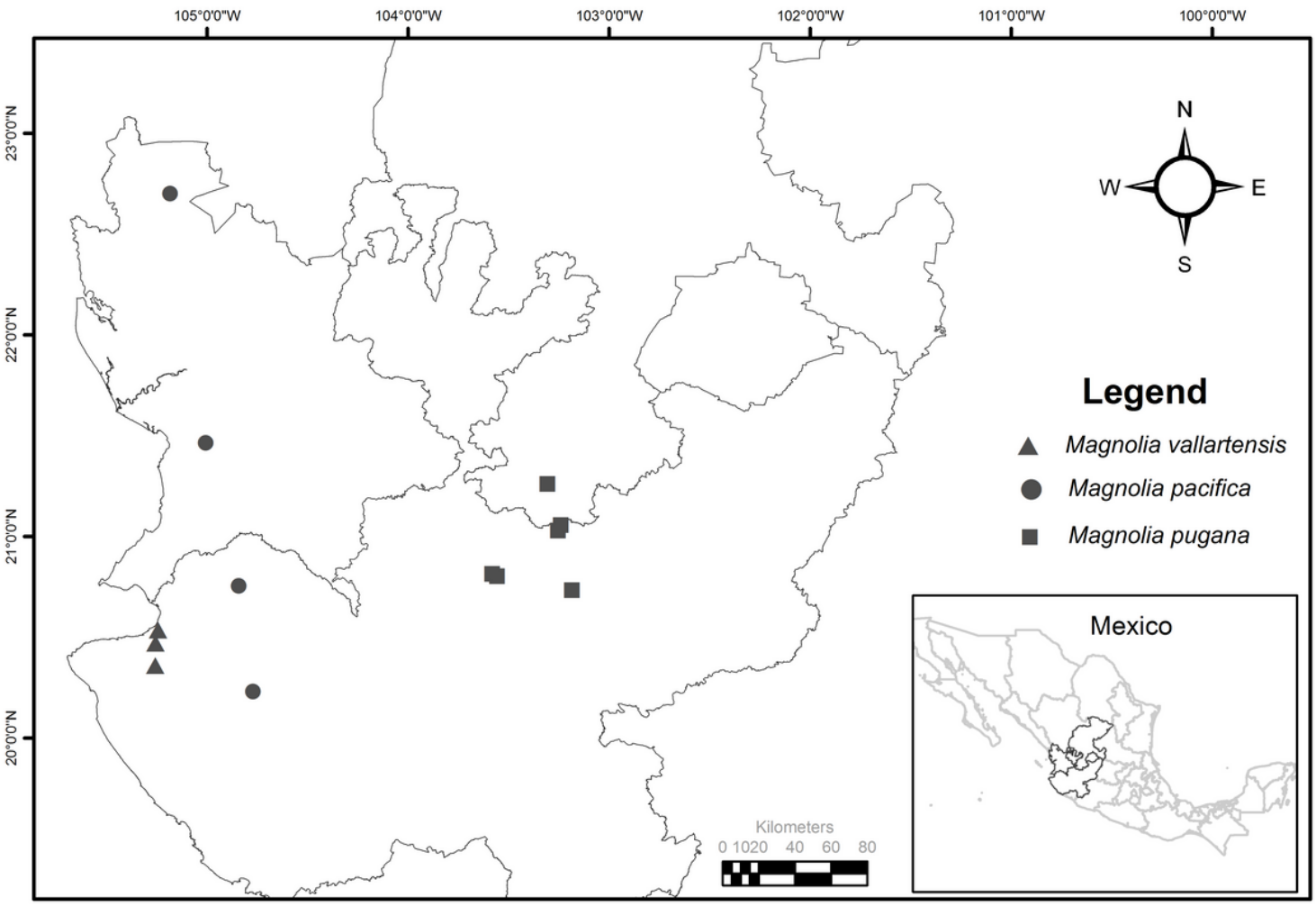

Figure 1

The geographic location of the 13 localities of three Magnolia species from western Mexico. Map elaborated based on a Geographic Information System Software ArcMap version 10.1 [70]. Note: The designations employed and the presentation of the material on this map do not imply the expression of any opinion whatsoever on the part of Research Square concerning the legal status of any country, territory, city or area or of its authorities, or concerning the delimitation of its frontiers or boundaries. This map has been provided by the authors.

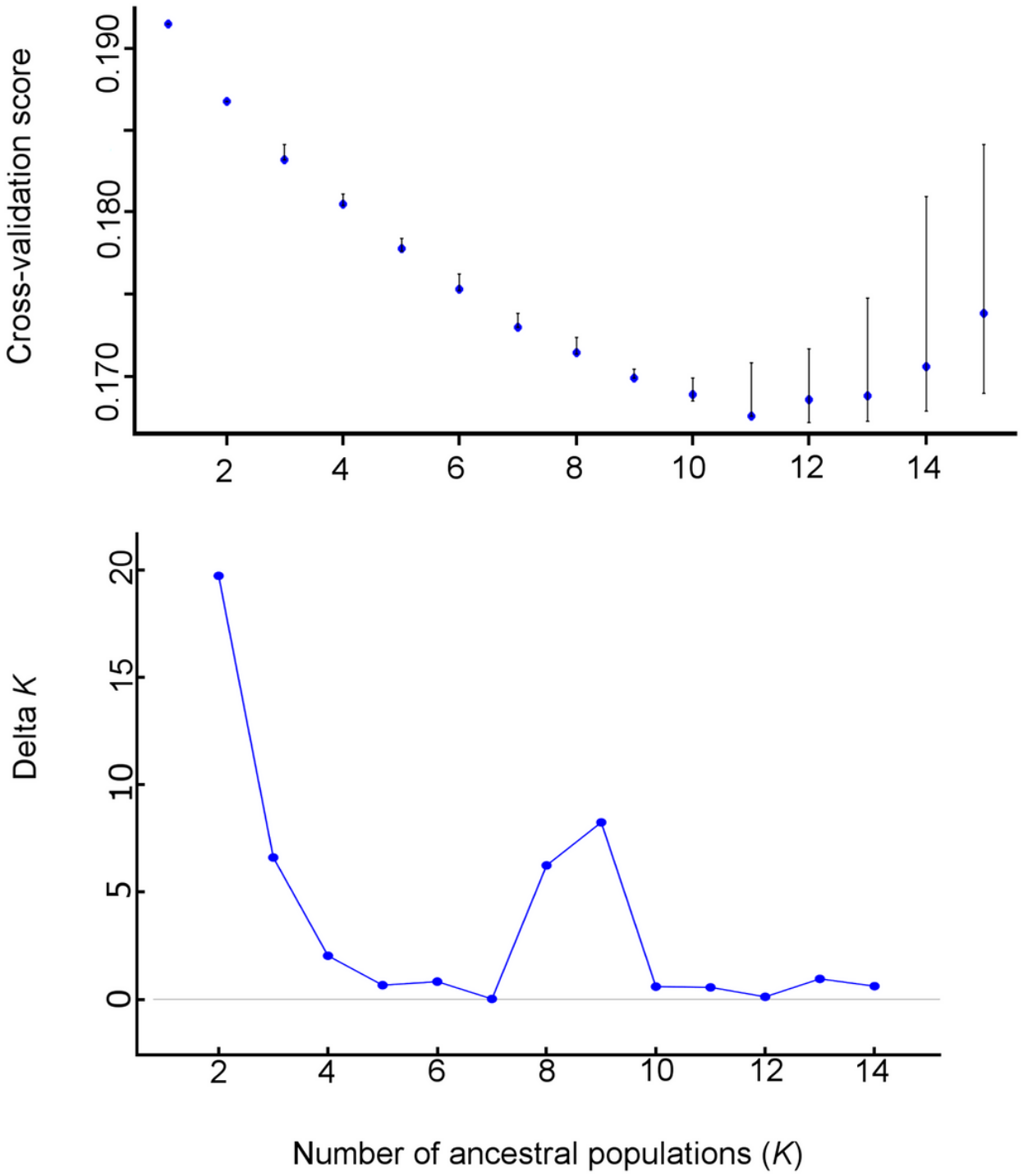

Figure 2

Plots from the cross-validation score (upper) for TESS 3 and the uppermost hierarchical level of the genetic partition by ΔK values (lower) for STRUCTURE.

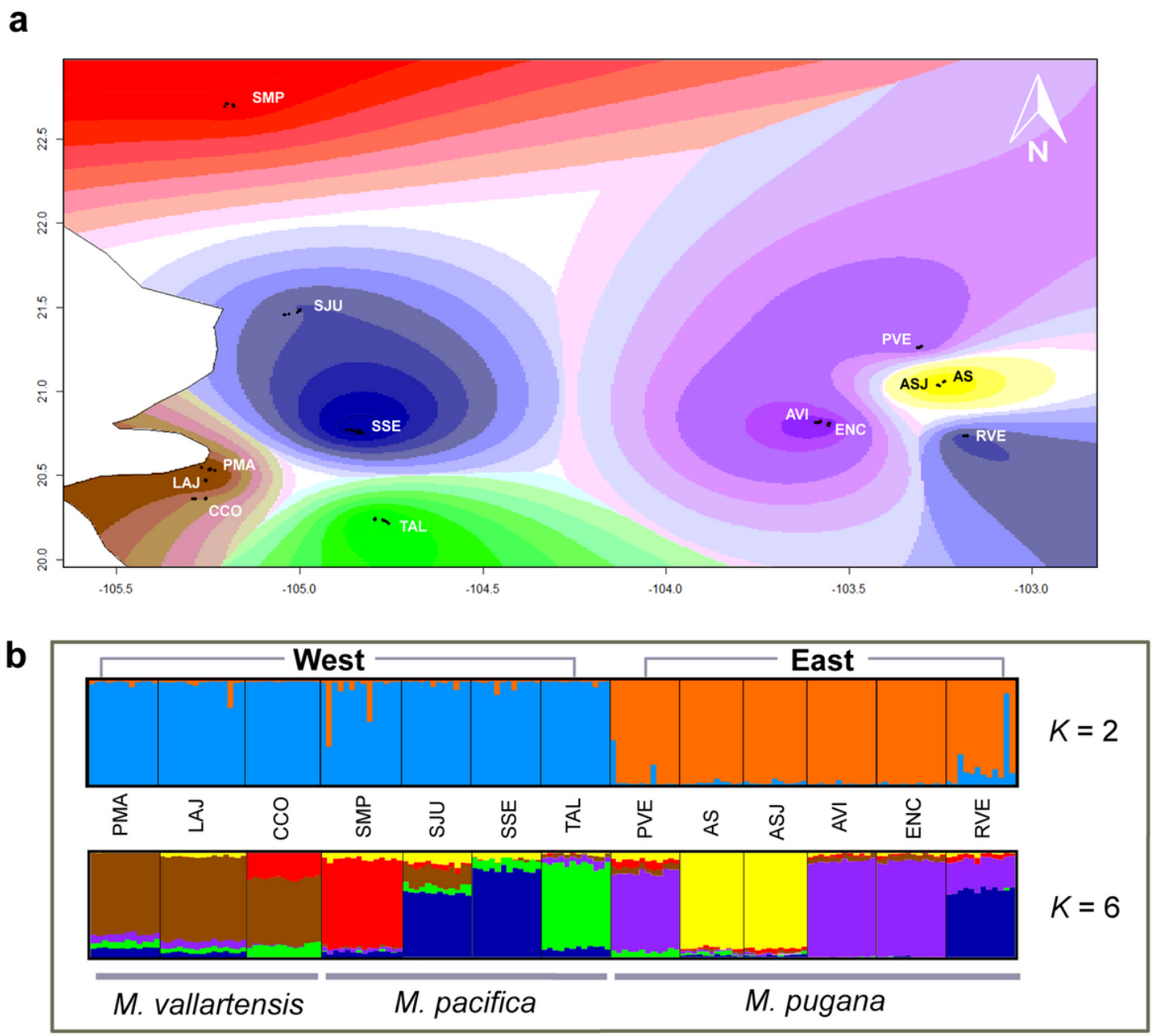

Figure 3

Clustering results from STRUCTURE and TESS 3. a) Geographic map of ancestry coefficient using K= 6 from TESS 3. b) The proportion of estimated ancestry using 160 individuals of three Magnolia species from the Mexican Pacific. The summary plot generated in CLUMPAK for STRUCTURE results for K= 2 (upper) showing west and east cluster; bar plot of TESS 3 for K= 6 (lower). Each individual is represented by a vertical line with the assignment probability to n number of clusters proportional to the length of each color. Populations IDs are as in Table 1.

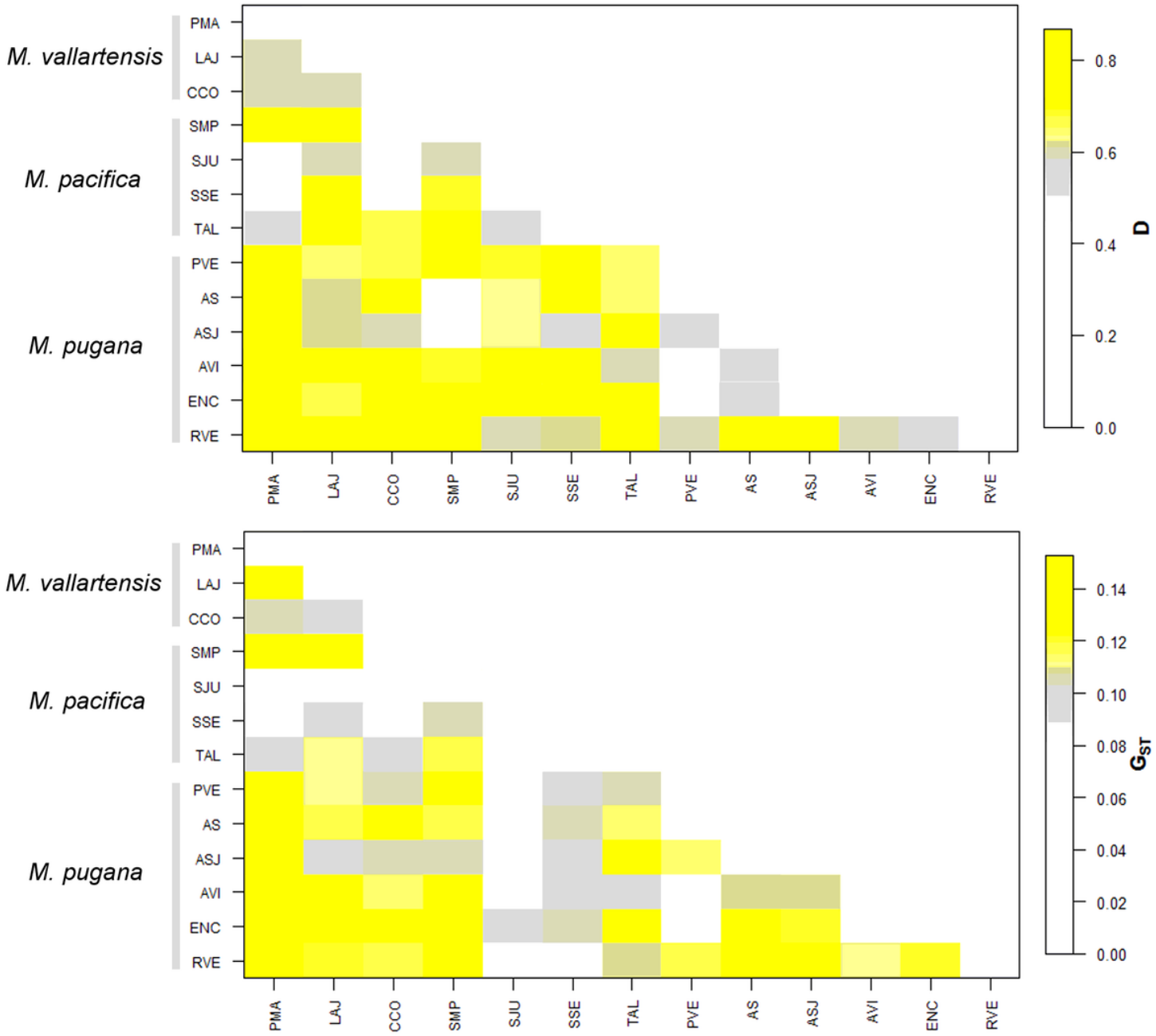

Figure 4

Pairwise D (upper) and GST (lower) values for 13 localities of three Magnolia species. Populations are abbreviated according to Table 1.

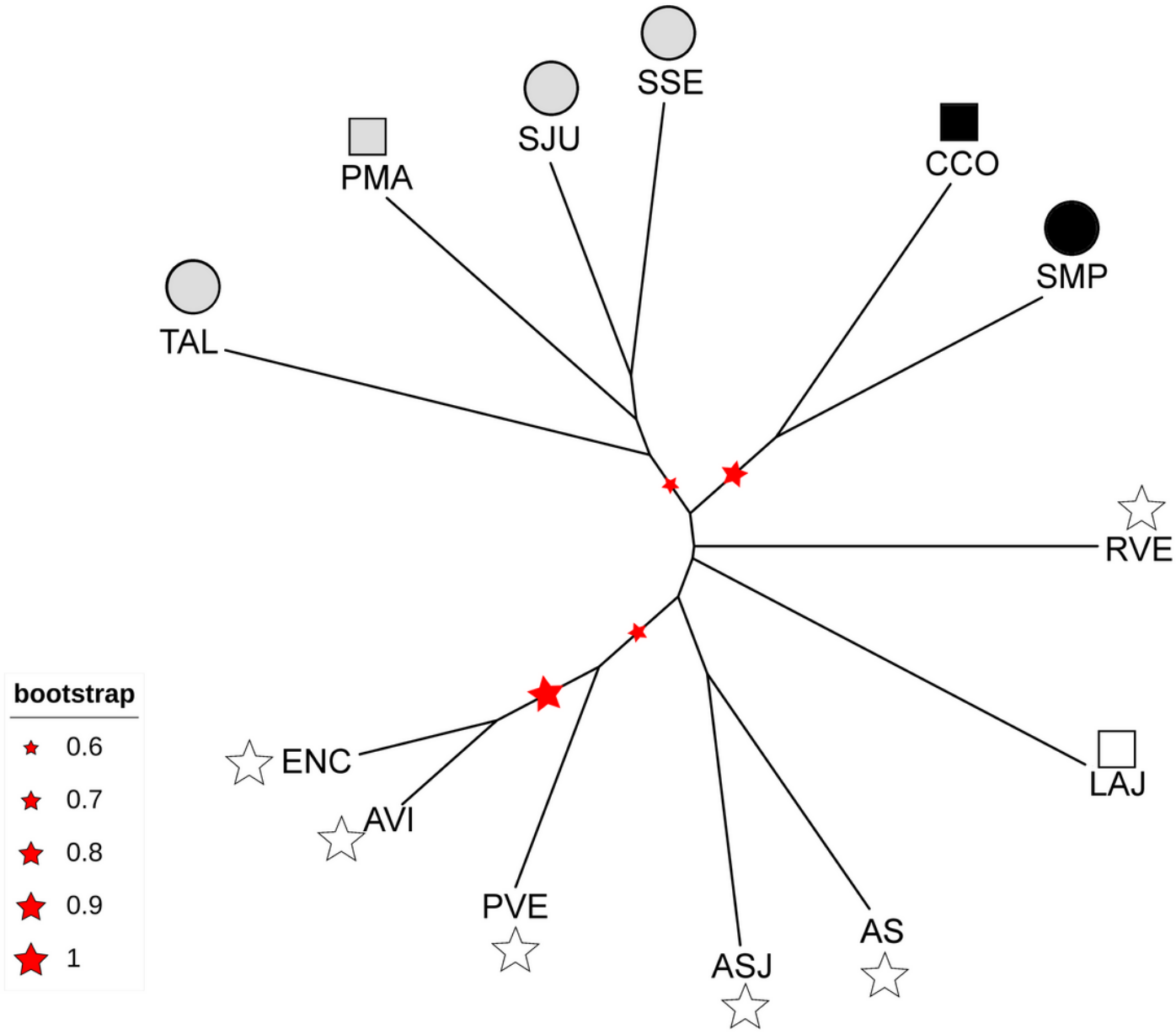

Figure 5

Phylogenetic relationships from 13 populations of three Magnolia species based on the neighbor-joining method. Different colors indicate three major lineages identified (gray, black, and white). We only showed bootstrap support of 60 % or higher. Squares, M. vallartensis; Circles, M. pacifica; stars, M. pugana; populations are abbreviated according to Table 1.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Appendix1Referencespecimens.docx
- SupplementarytablesS14andfigureS1.docx