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Genetic Variation in Two Rare Endemic Mexican Trees, *Magnolia sharpii* and *Magnolia schiedeana*

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Abstract

Patterns of genetic variation were examined within two endemic tree species restricted to Mexican cloud forest, *Magnolia sharpii* and *Magnolia schiedeana*. Leaf samples collected from natural populations were analysed using PCR RFLP of cpDNA, Inter-SSR and isozyme genetic markers, which were used to test a series of hypotheses regarding patterns of intraspecific variation within the two species. Genetic diversity estimates derived from Inter-SSR markers (mean S_{pop} of 0.56 and 0.50 for *M. sharpii* and *M. schiedeana* respectively) are comparable to values obtained for other tree species. As predicted on the basis of its larger geographic range, the degree of population differentiation was found to be higher within *M. schiedeana* than *M. sharpii*, with 12.9% and 3.4% of total variation recorded between populations for the two species respectively using isozymes, and 26% and 11% using Inter-SSR markers. Isozyme analyses indicated negative F_{is}

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values, which may be suggestive of inbreeding in populations of *M. sharpii*, but provided less evidence of inbreeding in *M. schiedeana*. On the basis of PCR RFLP analysis of cpDNA, two chloroplast types were differentiated, type A being recorded for all of the individuals of both species, with the exception of one population of *M. schiedeana* that was fixed for type B. These results are consistent with recent evidence suggesting that some endemic plant taxa are able to maintain relatively high diversity within populations despite the occurrence of inbreeding and genetic drift, and that species with wider geographic ranges tend to exhibit relatively high genetic differentiation among populations. Conservation strategies for these species need to take into account the significant genetic differences recorded among the populations studied.

Key words: cloud forest, conservation biology, genetic variation, isozyme, cpDNA, PCR-RFLP, *Magnolia sharpii*, *Magnolia schiedeana*.

Introduction

The objectives of the present investigation were to examine patterns of genetic variation within two threatened tree species endemic to Mexico, *Magnolia sharpii* Miranda and *Magnolia schiedeana* Schltdl. The genus *Magnolia* has a complex biogeographic history, belonging as it does to one of the oldest extant angiosperm families (Magnoliaceae) (DANDY, 1971). *Magnolia* is now distributed primarily in Asia (Nepal to New Guinea) and the Americas (E. Canada to Brazil and the Caribbean) (FRODIN and GOVAERTS, 1996). Both species considered here belong to section *Theorhodon* Spach., which based on Miocene fossil records, is thought to have contracted drastically in range over the last 25 million years owing to climatic change. A once continuous distribution that extended over much of Miocene USA and into Canada, Mexico and Central America has now been reduced to a highly disjunct distribution in SE USA, Mexico and Central America (FIGLAR, 1993).

A recent taxonomic revision of the Mexican and Central American species has resulted in the description of a number of new taxa (VÁZQUEZ, 1994), but as a group these trees are very poorly known (CALLAWAY, 1994). Following this revision, *M. schiedeana* now has a much smaller range than previously thought; it is apparently restricted to fourteen scattered populations ranging across the Mexican states of Querétaro, Hidalgo, Veracruz, Guerrero and Oaxaca (VÁZQUEZ, 1994). Populations typically occur on steep slopes or ravines in cloud forests at altitudes of 1500–2000 m (DIERINGER and ESPINOSA, 1994). *M. sharpii*, considered to be one of the most primitive species within the section, is endemic to the Mexican state of Chiapas (VÁZQUEZ, 1994), occurring at only five sites in evergreen cloud forests of the central highlands between elevations of 1950–2940 m (CALLAWAY, 1994). Both *M. schiedeana* and *M. sharpii* are considered as Endangered according to the IUCN Red List of Magnoliaceae (CICUZZA *et al.,* 2007).

The habitats of both of these species have been severely reduced in recent decades as a result of clearance for agriculture. Analysis of Landsat MSS satellite images supported by interpretation of aerial photography has indicated that the montane forests of Chiapas have been severely fragmented over the past 30 years (CAYUELA *et al.,* 2006), which has been accompanied by loss of floristic diversity (GONZÁLEZ-ESPINOSA *et al.,* 1991, 1995; GALINDO-JAIMES *et al.,* 2002). Similarly, in the state of Veracruz establishment of croplands, cattle ranching and the cutting of trees for fuelwood have been important causes of forest destruction, which severely threatens remaining forest fragments and their associated biodiversity (WILLIAMS-LINERA, 2002). *M. schiedeana* is used for medicinal purposes; for example a decoction of the flowers is used as a remedy for scorpion stings (CALLAWAY, 1994). Leaves of *M. sharpii* are used for wrapping tamales (a foodstuff); its timber was used to construct rifles during the Mexican revolution (M. MARTINEZ-ICÓ, pers. comm.).

Both species are evergreen trees reaching up to thirty meters in height (CALLAWAY, 1994). Very little is known about their ecology. *M. schiedeana* is known to be similar to most other members of the Magnoliaceae in being self-compatible and beetle pollinated (DIERINGER and ESPINOSA, 1994), although it seems to possess a more specialised pollination system than temperate *Magnolias*. One of its two known pollinators, *Cyclocephala jalapensis*, is a regionally endemic scarab beetle that appears to be dependent on *M. schiedeana* for adult nutrition and reproduction (DIERINGER and DELGADO, 1994). All *M. schiedeana* populations studied by DIERINGER and ESPINOSA (1994) exhibited very low fertility, which was attributed to previous episodes of overharvesting. In Chiapas, forest fragmentation and habitat modification of the remaining forest fragments appear to be limiting conditions for many understory trees, including *M. sharpii* (GONZÁLEZ-ESPINOSA *et al.,* 1991; RAMÍREZ-MARCIAL *et al.,* 2001).

The research was designed to test a series of hypotheses relating to the pattern of intraspecific variation within the two species. Specifically, given their contrasting patterns of distribution, and the fact that both species are restricted to relatively small forest fragments, based on current theory and empirical evidence from other species (NYBOM and BARTISH, 2000; HAMRICK *et al.,* 1992) we hypothesized that: (i) *M. schiedeana* maintains higher genetic variation within its populations compared with *M. sharpii* because of its wider geographic range; (ii) given their animal-pollinated breeding system, these species display relatively low intraspecific variation, but high population differentiation, compared with wind-pollinated tree species; (iii) higher within-population variation is expected within larger populations of both species; and (iv) geographically isolated populations are expected to be characterised by a relatively high degree of population differentiation. These hypotheses were tested using a variety of different genetic markers: PCR RFLP of cpDNA, inter-SSRs and isozymes.

Materials and Methods

Sample collection

The collection of material for analysis was limited by the rarity and restricted geographical ranges of the species under study. In the case of *M. sharpii*, samples

were collected from four out of the five sites in which the species is known to occur; the remaining site was not visited because of its inaccessibility (*Table 1, Figure 1*). In the case of *M. schiedeana*, samples were collected from each of the three populations known to exist in the vicinity of Xalapa, Veracruz (*Table 1, Figure 1*). These populations are isolated by substantial distances (> 100 km) from any other populations of the species, and form a cluster at a similar geographic scale to those of *M. sharpii*, facilitating comparison of the two species. Of the *M. sharpii* sample sites, sites 1–3 are clustered within less than 15 km of each other, while site 4 is located at a distance of 55–70 km from sites 1–3 (*Figure 1*). The three *M. schiedeana* populations are located within 25 km of each other: site 6 is located within 25 km of site 7, and site 5 is located within 10 km and 20 km of sites 6 and 7, respectively (*Figure 1*). The location of each site was recorded using a hand-held GPS receiv-

er (Silva, Livingston, UK) and altitude was recorded using a digital altimeter.

Samples were collected throughout the entire area of a site using a minimum distance between trees of 10 m to reduce the probability of sampling the same individual more than once. In each case, as many individuals were sampled as possible, which resulted in variation in sample size between sites (*Table 1*). Leaf material was collected from each individual sampled. Material for DNA analysis was dried and preserved in labelled, sealable plastic bags containing silica gel (S4883 silica, Sigma Chemical Company Ltd, Fancy Road, Dorset, UK) and stored at 4 °C prior to DNA extraction. For isozyme analysis, fresh leaf and bud material was placed in loosely sealed, 'Stayfresh longer' bags (Lakeland Limited, Windermere, Cumbria, UK) and stored in a refrigerator at 4 °C before isozyme extraction was carried out. DNA extractions were conducted according to a modified

Table 1. – Location and characteristics of (A) *Magnolia sharpii* and (B) *Magnolia schiedeana* populations sampled for analysis.

	A					
Site no.	Site name	Altitude (m)	Geographical coordinates (lat., long.)	Approx. area of population (ha)	No. sampled for Inter-SSR analysis	No. sampled for isozyme analysis
1	Merced-Bazom	2490	16°44'72"N, 92°28'58"W	15	16	36
2	Achlum	2400	16°46'58"N, 92°27'25"W	4	23	52
3	El Retiro	2070	16°49'11"N, 92°29'11"W	15	24	51
4	La Yerbabuena	2120	17°11'50"N, 92°53'79"W	150	17	29

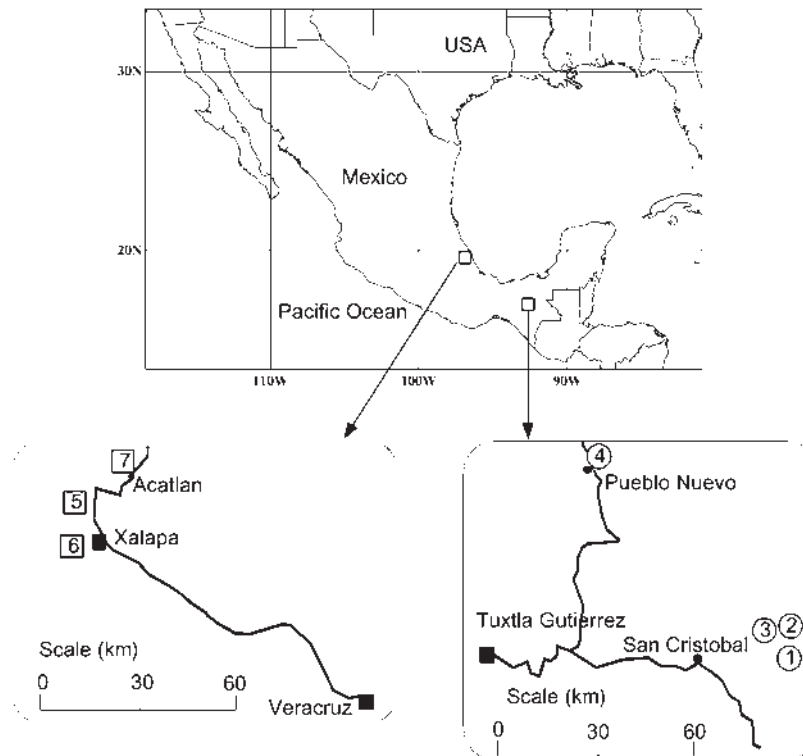|	B					
Site no.	Site name	Altitude (m)	Geographical coordinates (lat., long.)	Approx. area of population (ha)	No. sampled for Inter-SSR analysis	No. sampled for isozyme analysis
5	Banderilla	1520	19°34'97"N, 96°56'80"W	5	22	54
6	Acajete	1950	19°33'61"N, 97°01'05"W	15	20	54
7	Acatlán	1810	19°40'78"N, 96°51'20"W	5	22	44

Figure 1. – Distribution of populations of *Magnolia sharpii* (populations 1–4) and *M. schiedeana* (populations 5–7) included in the genetic analysis.

procedure of the CTAB method, as described previously (ALLNUTT *et al.,* 1999).

PCR-RFLP of cpDNA

A set of six universal primer-pairs was used to amplify non-coding regions of *Magnolia* chloroplast DNA (c-d and e-f, TABERLET *et al.,* 1991; A, B, E and H, DEMESURE *et al.,* 1995). Analyses of cpDNA were performed on a subsample of four individuals selected at random from the sample population collected from each site.

PCR reaction mixtures (20 µl total volume) contained the following components/concentrations: one unit of Taq DNA polymerase (Bioline), 10x reaction buffer (Bioline), 1.5–3.5 mM $MgCl_2$ (Bioline), 200 µM of each dNTP (Sigma), with 0.2 µM of each forward and reverse primer and approx. 50 ng of genomic DNA. PCR reactions were carried out in a Perkin-Elmer 480 thermal cycler, in thin-walled 0.6 ml reaction tubes. The following programme was used: 1 step at 94°C for 2 min, followed by 35 cycles of 1 min at 94°C, 1 min at 50–58°C, 2 min at 72°C, and a final 5–7 min extension step at 72°C.

Restriction digest reaction mixtures contained the following components: 2 µl of restriction enzyme10x reaction buffer (Promega), 2 µl of BSA (10 mg ml^{-1}), 2 µl of restriction enzyme (10 µg $µl^{-1}$), 8 µl of DNA (PCR sample), made up to a final volume 20 µl. Samples were mixed gently by pipetting, centrifuged briefly and incubated overnight at 37°C or 65°C for *Taq* I. Restriction digest products were separated by vertical gel electrophoresis using 6% native polyacrylamide gels, run at 400v for 1.5 hours.

To identify suitable primer-pair enzyme combinations with the potential to detect chloroplast DNA polymorphism among *Magnolia* individuals, four individuals of each species were screened with the above primer-pairs. The PCR products were subsequently digested with a set of eight restriction enzymes (Promega *Alu* I, *Cfo* I, *Rsa* I, *Hsp*92 II, *Hae* III, *Hpa* II, *Hinf* I and nbl Gene Sciences *Taq* 1).

Inter-SSRs

Inter-simple sequence repeats (Inter-SSRs) use primers with an anchor short sequence (3–4 base pairs) to amplify variable microsatellites or flanking regions. This method examines random loci in much the same way as RAPDs, but is thought to give more reproducible markers than RAPDs (NEWTON *et al.,* 1999). DNA samples of 80 *M. sharpii* individuals sampled from four populations, and of 64 *M. schiedeana* individuals sampled from three populations (*Table 1*) were analysed using Inter-SSR primers, following the method described by CHARTERS *et al.* (1996). Sample sizes were limited by the poor quality of the DNA obtained from a small proportion of the leaf samples, and consequently sample sizes were smaller than for the isozyme analysis. 34 Inter-SSR primers, set 9, obtained from the University of British Columbia were screened for their ability to produce reliable DNA markers. From these, a subset of eight primers (811, 820, 834, 841, 843, 849, 851, and 855; *Table 2*) was used to screen *M. sharpii* populations and a subset of seven primers (811, 820, 834, 836, 841, 843 and 855) was used to screen *M. schiedeana* (*Table 2*).

Primer	Sequence
811	$(GA)_8 C$
820	$(GT)_8 C$
834	$(AG)_8 YT$
836	$(AG)_8 YA$
841	$(GA)_8 YC$
843	$(CT)_8 YT$
849	$(GT)_8 YA$
851	$(GT)_8 YG$
855	$(AC)_8 YG$

PCR reaction mixtures (20 µl) contained the following components/ concentrations: 1 unit of Taq DNA polymerase (Bioline), 10 x reaction buffer (Bioline), 1.5 mM (MgCl$_2$), 0.2 mM of each dNTP (Sigma), with 0.2 µM of each primer and 20–50 ng of DNA. PCR reactions were carried out in a Perkin-Elmer 480 thermal cycler, in thin walled 0.6 ml reaction tubes. The following programme was used: 1 step at 95 °C for 3 min, followed by 30 cycles of 1 min at 94 °C, 1 min at 55 °C, 1 min at 72 °C, and a final 5-min extension step of 72 °C. PCR amplification products were separated on 1.5 % agarose gels and visualised by staining with ethidium bromide and photographed over UV light.

PCR products from each primer were scored for each individual and each band was recorded as present (1) or absent (0). These data were entered into a binary matrix and a simple pairwise distance measure was calculated (JACCARD, 1908). These distances were used to perform analysis of molecular variance (AMOVA) (EXCOFFIER *et al.,* 1992). Shannon's diversity estimates (LEWONTIN, 1972) were calculated to provide a relative estimate of the degree of variation within each population according to the formula: $S = -\Sigma p_i \log_2 p_i$, where p_i is the frequency of presence or absence of each band (treating each band as a single locus according to LEWONTIN, 1972). S$_s$

was calculated as the mean value of S within all of the populations sampled.

Isozyme analyses

Isozyme analyses were performed following standard procedures (PARKS *et al.,* 1990; CHELIAK and PITEL, 1984). Lithium borate gels with 34.98g:1.74g:270 ml of starch:sucrose:gel by volume, and morpholine-citrate (CN-3-M) gels with 34.99:8.10g:270 ml of starch:sucrose:gel by volume were used for electrophoresis as these had previously been used successfully with other members of the Magnoliaceae (QUI and PARKS, 1994). Anodal slices of the gels were cut horizontally and activity of the following enzymes was stained for, following the procedures described by SOLTIS *et al.* (1983): Asparate aminotransferase (AAT), Phosphoglucose isomerase (PGI), Shikimate dehydrogenase (SDH) and 6-Phosphogluconate dehydrogenase (6-PGD). These were selected on the basis that they detected polymorphism during preliminary screening. Knowledge of the monomeric or dimeric structure of these enzymes was used to infer isozyme genotypes from observed banding patterns. Two alleles were detected at each of the loci scored. The FSTAT program (FSTAT 2.9.3 (2002) J. Goudet, UNIL, Switzerland) was used to estimate Fis, to indicate any deficit or excess of heterozygotes, and to estimate gene diversity for each population.

Results

PCR-RFLP of cpDNA

Samples were amplified with six chloroplast primer-pairs; primer-pairs CD, EF (TABERLET *et al.,* 1991) AB, and EH (DEMESURE *et al.,* 1995) produced single bands that were clear, reproducible and suitable for subsequent restriction digest reactions. Restriction digest banding patterns for primer-pairs EF and AB were monomorphic for each of the above restriction enzymes. Banding patterns for primer-pairs CD and EH showed polymorphism when digested with restriction *Alu* I, *Hae* III, *Hinf* I *Taq* 1 *Rsa* I and *Hpa* II. On the basis of these results, two chloroplast types were differentiated, denoted types A and B. Chloroplast type A was recorded

Table 3. – AMOVA analysis of Inter-SSR variation of (A) *Magnolia sharpii* and (B) *Magnolia schiedeana.*

A

	df.	Sum of squares	Mean squares	Variance component	% of total variance	P
Among pop.	3	1.89	0.63	0.02	10.58	0.002
Within pop.	76	14.34	0.19	0.19	89.42	

B

	df.	Sum of squares	Mean squares	Variance component	% of total variance	P
Among pop.	2	3.12	1.56	0.06	25.68	0.002
Within pop.	61	11.38	0.18	0.19	74.32	

for all of the individuals of both species with the exception of population 6 (*M. schiedeana*), which was fixed for chloroplast type B.

Inter-SSRs

A total of 20 polymorphic bands were scored for *M. sharpii*. From these 20 bands a total of 79 genotypes was recognised with individuals 7 and 24 sharing the same banding pattern. For *M. schiedeana* a total of 17 polymorphic bands were scored and from these bands a total of 53 genotypes were recorded with individuals 5 and 6; 15 and 22; 29 and 37; 31 and 40; 46, 54, and 63; 47 and 64; 48, 51, 58 and 61; 52 and 59 each sharing the same banding pattern.

The results of the AMOVA for *M. sharpii* showed that most of the genetic variation was found within populations (89%, *Table 3a*). However, a significant proportion (11%, P < 0.002) was attributed to differences among populations. All populations differed significantly from each other (P < 0.05), with the exception of populations 1 and 3, with the highest pairwise Φ_{ST} value (0.21) recorded between populations 1 and 4. For *M. schiedeana*, 74% of the genetic variation was found within populations and 26% found among populations (P < 0.002) (*Table 3b*). All populations were significantly different from each other (P < 0.05), with the highest pairwise Φ_{ST} value (0.30) recorded between populations 5 and 7.

The relative diversity within each *M. sharpii* population, as measured by Shannon's index, was calculated as 0.50 for population 1, 0.53 for population 2, 0.69 for population 3 and 0.49 for population 4, with a mean S_s of 0.56. For *M. schiedeana* populations, values of Shannon's index were recorded as 0.59 for population 5, 0.65 for population 6 and 0.34 for population 7, with a mean S_s of 0.50.

Isozymes

All of the resolved putative isozyme loci were polymorphic in all of the populations, with two alleles recorded per locus in each case. Allele frequencies differed markedly between populations; for example, for PGI-1 frequencies of allele A ranged from 0.33 (population 2) to 0.74 (population 5). In the case of 6PGD-1, frequencies of allele A for populations of *M. sharpii* ranged from 0.5–0.6; those recorded for *M. schiedeana* displayed a much wider range, from 0.25 (population 5) to 0.94 (population 6). Frequencies of SDH-1 displayed little variation between populations, values for allele A ranging from 0.52 (population 7) to 0.72 (population 5). In the case of AAT-1, populations 1 and 2 displayed relatively low frequencies of allele A (0.26 and 0.375 respectively); all other values were above 0.65. In general, populations 3 and 4 displayed similar allele frequencies, whereas population 5 displayed a markedly different profile of allele frequencies than the other populations sampled.

Estimates of F_{is} based on isozyme data indicate that all populations of *M. sharpii* have an excess of heterozygotes (F_{is} values very negative), providing some evidence of inbreeding (*Table 4*). The F_{is} value for population 5 of *M. schiedeana* also provided some evidence of inbreeding; other populations of this species were close to ran-

Table 4. – Estimates of F_{is} and gene diversity for populations of (A) *Magnolia sharpii* and (B) *M. schiedeana*, based on isozyme data. For methods of calculation, see text. For details of populations, see *Table 1*.

A. *M. sharpii*
(i) F_{is} per population

	Population number			
	1	2	3	4
PGI-1	0.06	0.27	-0.32	0.19
6PGD-1	-0.68	-1.00	-0.86	-0.68
SDH-1	-0.40	-0.22	-0.59	-0.25
AAT-1	0.12	0.18	-0.49	0.03
All	-0.23	-0.21	-0.57	-0.17

(ii) Expected heterozygosity (gene diversity)

	1	2	3	4
PGI-1	0.49	0.45	0.49	0.51
6PGD-1	0.49	0.50	0.50	0.49
SDH-1	0.43	0.43	0.47	0.40
AAT-1	0.39	0.48	0.45	0.46
Mean	0.451	0.46	0.478	0.46
95%CI	0.046	0.03	0.022	0.05

(B). *M. schiedeana*
(i) F_{is} per population:

	Population number		
	5	6	7
PGI-1	-0.33	0.30	0.286
6PGD-1	0.63	0.66	0.138
SDH-1	-0.35	-0.26	-0.457
AAT-1	-0.14	-0.23	0.161
All	-0.05	0.01	0.003

(ii) Gene diversity per locus and population:

	5	6	7
PGI-1	0.39	0.46	0.50
6PGD-1	0.39	0.11	0.26
SDH-1	0.40	0.45	0.51
AAT-1	0.38	0.32	0.35
Mean	0.39	0.33	0.40
95%CI	0.01	0.16	0.12

dom mating (F_{is} values close to 0). Estimates of G_{st} based on isozyme data provided values of 0.034 and 0.129 for *M. sharpii* and *M. schiedeana* respectively, indicating a higher degree of population differentiation within the latter species. Estimates of gene diversity highlighted little difference between populations of either species, although values recorded for *M. sharpii* tended to be higher than those recorded for *M. schiedeana* (*Table 4*).

Discussion

As both *M. sharpii* and *M. schiedeana* are endemic species with restricted ranges, we hypothesized that both species would display relatively low genetic variation overall. However, the current results do not support this contention. Measures of the extent of within-population variation assessed using the Inter-SSR markers (mean S_{pop} of 0.56 and 0.50 for *M. sharpii* and *M. schiedeana* respectively) are comparable to values obtained for other tree species using RAPD markers, for

example 0.54 for *Pinus chiapensis* (NEWTON *et al.,* 2002), 0.57 for *Pilgerodendron uviferum* (ALLNUTT *et al.,* 2003), 0.43 for *Cedrela odorata* (GILLIES *et al.,* 1997), 0.55 for *Fitzroya cupressoides* (ALLNUTT *et al.,* 1999), 0.65 for *Araucaria araucana* (BEKESSY *et al.,* 2002) and 0.36 for *Swietenia macrophylla* (GILLIES *et al.,* 1999). Interestingly, the value recorded for *Fagus grandiflora* var. *mexicana*, another endemic Mexican tree restricted to cloud forests, was substantially lower (0.20) (ROWDEN *et al.,* 2004).

As noted by NYBOM and BARTISH (2000), a number of studies have reported relatively high within-population genetic diversity for endemic plant taxa, suggesting that some species are able to maintain relatively high genetic variability despite the higher risk of genetic drift and inbreeding in small, isolated populations. The current results suggest that this may be the case for the *Magnolia* species studied here. However, measures of within-population variation in both species appeared to be unrelated either to the size or to the degree of isolation of the forest fragment. These results contrast markedly with those obtained for *Fagus grandiflora* var. *mexicana*, where within-population variation was found to be significantly correlated with population size (ROWDEN *et al.,* 2004).

Based on the review of NYBOM and BARTISH (2000), we expected that *M. sharpii* would be characterised by a lower degree of population differentiation than *M. schiedeana*, given that the latter species has a substantially wider geographic range. Based on the results of the isozyme analysis, the degree of population differentiation recorded here within *M. schiedeana* (12.9%) appears to be typical for a species with its ecological characteristics. A review of isozyme studies undertaken with 73 woody species of angiosperm indicated that on average, 10.2% of genetic variation was recorded between rather than within populations; when endemic species were considered as a group, a mean value of 14.1% was recorded (HAMRICK *et al.,* 1992). The mean value for woody species with a mixed-animal breeding system was 12.2% (HAMRICK *et al.,* 1992). In contrast, the extent of population differentiation recorded here within *M. sharpii* was relatively low (3.4%). Values of population differentiation recorded using Inter-SSR markers were consistent with these results, with lower values recorded for *M. sharpii* than *M. schiedeana*, supporting our initial hypothesis (11% and 26% for the two species respectively).

This finding supports the findings of NYBOM and BARTISH (2000), as the species with wider geographic range exhibited higher differentiation among populations. However, the results contrast with the conclusions of HAMRICK *et al.* (1992). In particular, the low degree of population differentiation recorded for *M. sharpii* is surprising. This implies that gene flow is occurring between populations of the species, or has occurred in the past, despite the fragmented nature of the landscapes in which they occur. However, the results from the isozyme analysis provide some evidence for possible inbreeding in this species, based on the presumed removal of homozygotic individuals. In the absence of information

about the mating system or investigations of controlled crosses, the evidence of inbreeding must be considered as tentative. Very little is known about the ecology of *M. sharpii*; it is conceivable that it is dependent on specialised beetles for pollination, as recorded for *M. schiedeana* (DIERINGER and ESPINOSA, 1994), but this is unknown at present. Alternatively, as the seeds are bird-dispersed, it is conceivable that gene flow between forest fragments is occurring through movement of seed dispersal vectors. Recent assessments of size structure distribution suggest that populations of *M. sharpii* are regenerating (RAMÍREZ-MARCIAL *et al.,* 2001), but the germinability of seeds appears to be very variable (N. RAMÍREZ-MARCIAL, personal observation). Research into patterns of gene flow in two Japanese *Magnolia* species using microsatellite markers has recorded pollen movement over distances of 540 m, and long-distance gene flow resulting from bird dispersal, but has also recorded inbreeding depression as a result of differences in flowering phenology (ISAGI *et al.,* 2000), and a high degree of population differentiation in one species (KIKUCHI and ISAGI, 2002), as recorded here.

Isozyme analyses provide less evidence for inbreeding within *M. schiedeana*. However, the degree of population differentiation recorded here for this species using Inter-SSR markers is exceptionally high compared to studies of other tree species using similar approaches. For example, the Φ_{ST} value recorded here (0.26) was substantially higher than values derived from RAPD analysis of wind-pollinated trees Araucaria araucana (0.13, BEKESSY *et al.,* 2002), *Fitzroya cupressoides* (0.14, ALLNUTT *et al.,* 1999), *Podocarpus salignus* (0.07, ALLNUTT *et al.,* 2001), *Pilgerodendron uviferum* (0.19, ALLNUTT *et al.,* 2003), *Pinus chiapensis* (0.23, NEWTON *et al.,* 2002) and *Fagus grandifolia* var. *mexicana* (0.16, ROWDEN *et al.,* 2004). This result implies limited gene flow between populations of *M. schiedeana*, either currently or historically.

A particularly noteable result was the cpDNA polymorphism recorded for *M. schiedeana* in Acatlán. The occurrence of a cpDNA polymorphism within such a small geographic area contrasts with results from widespread tree species; for example in 64 populations of *Liriodendron tulipifera* (Magnoliaceae) distributed throughout the eastern USA, only two cpDNA haplotypes were recorded (SEWELL *et al.,* 1996). Similarly in European beech (*Fagus sylvatica*), much of central Europe was found to be dominated by a single cpDNA haplotype, interpreted as indicating postglacial recolonization from a single forest refugium in south-east Europe (DEMESURE *et al.,* 1996). Intriguingly, one of the three haplotypes recorded for another endemic tree, Mexican beech (*Fagus grandiflora* var. *mexicana*), was also found in Acatlán (ROWDEN *et al.,* 2004). This result, coupled with the high degree of population differentiation recorded by Inter-SSR markers suggests that the *M. schiedeana* populations studied here may have distinct biogeographic histories, despite their relatively close proximity.

The pronounced climatic changes experienced in Mexico during the Holocene (from around 8500–9000 yr BP)

had a major impact on forest distribution and composition (HEINE and OHNGEMACH, 1976), and may have led to the fragmentation and restriction of the cloud forests in which *Magnolia* currently occurs. Mexican cloud forests, which are today fragmented throughout their range, are remnants of a 'temperate rain forest' that extended to the eastern United States during the early Tertiary (GRAHAM, 1973; 1976). Those populations characterised by different cpDNA haplotypes may have been isolated for a substantial period of time (ENNOS *et al.,* 1999). However, further analyses are required to test this hypothesis more fully.

Given their complex biogeographic history, the relative impacts of recent human activities on genetic variation in these species are difficult to elucidate. The cloud forest habitat in which both *M. sharpii* and *M. schiedeana* occur is undoubtedly under severe pressure, primarily from clearance for agriculture (WILLIAMS-LINERA, 2002; NEWTON, 2007). Recent observations in the Chiapan cloud forests in which *M. sharpii* occurs suggest that floristic diversity is declining rapidly as a result of frequent, small-scale anthropogenic disturbance (RAMÍREZ-MARCIAL *et al.,* 2001; GALINDO-JAIMES *et al.,* 2002; NEWTON, 2007). A similar situation prevails in the forests in Veracruz where *M. schiedeana* occurs (WILLIAMS-LINERA, 2002). As both species now appear to be severely restricted in distribution, they both must be considered at severe risk of extinction, and as high priorities for conservation action.

The analyses of genetic variation presented here could inform the development of conservation strategies by assisting in the definition of appropriate units for conservation, as proposed for other tree species (NEWTON *et al.,* 1999). The fact that significant genetic differences were recorded here between most of the populations studied suggests that most, if not all, of the remaining populations of these species should be afforded protection in order to adequately conserve the full range of intraspecific variation that exists. Future research might usefully focus on confirming whether or not inbreeding is occurring within the species, and estimating current rates of gene flow in these species using microsatellite markers (ISAGI *et al.,* 2000; KIKUCHI and ISAGI, 2002), enabling the relationship between current patterns of land use and the viability of fragmented populations to be analysed.

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