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Chloroplast DNA microsatellites reveal contrasting phylogeographic structure in mahogany (*Swietenia macrophylla* King, Meliaceae) from Amazonia and Central America.

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## ABSTRACT

Big-leaf mahogany (*Swietenia macrophylla* King) is one of the most valuable and overharvested timber trees of tropical America. In order to better characterize geographic patterns of genetic variation, we performed a phylogeographic analysis of *S. macrophylla* based on six polymorphic chloroplast genome simple sequence repeat loci (cpSSRs) analyzed in 16 populations (N = 245 individuals) distributed across Central America and the Brazilian Amazon. Of the 31 total cpDNA haplotypes identified, 16 occurred in Central America and 15 in Amazonia with no single haplotype shared between the two regions. Populations from Central America showed moderate differentiation ( $F_{ST} = 0.36$ ) while within population genetic diversity was generally high (mean Nei's  $H_E = 0.639$ ). In contrast, the Amazonian populations were strongly differentiated ( $F_{ST} = 0.91$ ) and contained relatively low genetic diversity (mean  $H_E = 0.176$ ), except for one highly diverse population ( $H_E = 0.925$ ) from eastern Amazonia. Spatial analysis of molecular variance (SAMOVA) identified a single Central American phylogroup and four Amazonian phylogroups, indicating stronger phylogeographic structure within Amazonia. The results demonstrate distinctive regional patterns of *S. macrophylla* differentiation, and the first evidence of a strong phylogeographic break between Central American and South American mahogany populations. We suggest that the frequent occurrence of hurricanes in Central America, the differences in the glacial histories and in the duration and intensity of anthropogenic disturbance during the late Holocene may have played important roles in the geographic structuring of cpDNA lineages in the two regions. The high private haplotype diversity in Brazilian populations suggests that cpSSRs can be used as DNA barcodes for regional timber certification.

Key words: Amazon basin, Mesoamerica, tropical trees, big-leaf mahogany, cpSSRs, phylogeography, DNA barcodes, SAMOVA.

## INTRODUCTION

Mahogany, *Swietenia macrophylla* King (Meliaceae), is the most valuable hardwood species in Neotropics and is seriously threatened owing to over-exploitation and habitat destruction. *Swietenia macrophylla* has a wide geographic range from Mexico through Central America and across the southern arc of the Amazon basin in Bolivia and Brazil (Lamb, 1966; Pennington, 1981). The species has wide ecological tolerance and occurs in a variety of habitats from wet to seasonally dry, evergreen to deciduous, tropical to subtropical forests, with typically 800 - 2,500 mm of annual rainfall and at altitudes ranging from sea level to 1,400 m (Lamb, 1966; Whitmore, 1983). However, the species reaches its optimum natural development in Holdridge's (1971) tropical dry forest formation (Lamb, 1966). Mahogany tends to occur in widely scattered patches and its density within patches is typically less than one commercial-size tree per hectare (Whitmore, 1983; Verissimo et al., 1995). This patchy distribution is probably related to its mode of regeneration that requires major disturbances such as river course changes, hurricanes, blowdowns, and fire (Snook, 1996). In these situations, stands may be comprised of one or a few cohorts (Grogan et al., 2003).

*Swietenia macrophylla* has been exploited throughout its natural range since the beginning of the 20<sup>th</sup> century (Lamb, 1966; Rodan et al., 1992). In recent decades, with the depletion of natural stands in Central America, most of the extraction has come from populations in South America, especially in the Brazilian Amazon. Mahogany extraction is based on selective logging, which usually removes only the tallest trees of good form and with a dbh (diameter at breast height) > 80 cm (Verissimo et al., 1995;

Gullison et al., 1996). In addition to removing the most fecund trees, selective logging may have a significant impact on genetic structure and population size, and compromise the evolutionary viability of natural mahogany populations (Cornelius et al., 2005). The inclusion of *S. macrophylla* in CITES Appendix II in 2002 aimed to control international trade by ensuring that logging will not be detrimental to the survival of the species (Grogan and Barreto, 2005).

Studies of the organization of genetic diversity of *S. macrophylla* have been carried out in the Brazilian Amazonia (Lemes et. al., 2003) and in Central America (Novick et al. 2003) using nuclear microsatellite DNA markers. The studies sampled populations across a similar spatial scale (ca. 1600 km in Central America; 2103 km in Amazonia) using seven loci in common. Both studies showed significant isolation by distance patterns, and moderate levels of population differentiation ( $R_{ST}$ ). Furthermore, the Central American populations exhibited significantly lower mean genetic diversity than the Amazonian populations, which Novick et al. (2003) suggested may have resulted from the smaller, more dissected nature of suitable habitat in Central America, combined with more severe vegetation changes during the glacial phases of the Pleistocene.

A phylogeographic approach based on chloroplast genome (cpDNA) variation can provide additional insight into the historical patterns of genetic divergence across the range of *S. macrophylla*. Chloroplast DNA is a haploid genome and is maternally inherited in the majority of the angiosperms (Birky, 1995; McCauley 1995; Ennos et al 1999). Because of its four-fold smaller effective population size, chloroplast markers can often detect geographic structure that is not apparent in nuclear DNA markers (Cavers et al., 2003; Petit, 2005). Unfortunately the relatively low rates of nucleotide substitution in the chloroplast genome (Wolfe et al., 1987) have often impeded its use in

phylogeographic studies (Schaal, 1998). However, highly variable mononucleotide repeat loci in the chloroplast genome (cpDNA microsatellites or simple sequence repeats [cpSSRs]) have provided a rich source of variation for studies of phylogeography and gene flow (Provan et al., 2001).

In addition to its utility for phylogeographic studies, conservation and management purposes, cpSSRs may be useful as regionally distinct cpDNA barcodes that could permit forensic verification of timber origins (Deguilloux et al., 2002; Dick and Kress, 2009). Here we report on the phylogeographic structure of *S. macrophylla* populations sampled in Central America (Novick et al. 2003) and Amazonia (Lemes et al. 2003) based on cpDNA microsatellites. The main aims of the study were: (1) to evaluate the utility of chloroplast microsatellite loci for assessing intraspecific variation in *S. macrophylla*; (2) to quantify and compare the organization of genetic diversity of *S. macrophylla* populations in Central America and Amazonia ; and (3) to determine if population-specific cpDNA haplotypes are credible as regional DNA barcodes for monitoring timber harvests.

## RESULTS

### Genetic Variation

Six out of 10 cpSSR loci initially assayed using universal primers (Weising and Gardner, 1999) successfully amplified and were found to be polymorphic. All individuals (n = 245) from eight Central American and eight Amazonian populations were analyzed for these six polymorphic cpSSR loci (ccmp 2, ccmp 3, ccmp 4, ccmp 5, ccmp 7, ccmp 10).

A total of 31 different haplotypes and 30 cpSSR alleles were found. The composition of the haplotypes and their distribution in the populations are given in

Table 1. The number of size variants (alleles) per locus varied from three to six. Gene diversity indices ( $H_E$ ) showed a high range of variation across populations (0.000 to 0.925) (Table 2).

### **Structuring and geographical distribution of haplotypes**

The hierarchical analysis of genetic variation within and among populations performed for each geographical region (Amazonia and Central America) showed contrasting patterns. Most of the variation found in Amazonia was partitioned among populations (91%), while in Central America most variation was partitioned within populations (64%, Table 3).

The pattern of cpSSR haplotype organization provides evidence of a strong phylogeographic break between *S. macrophylla* populations in Central America and Amazonia. Of the 31 haplotypes detected, 15 occurred exclusively in Amazonian populations and the remaining 16 in Central America (Table 2), with no single haplotype shared between the two geographical regions. A maximum parsimony median-joining network (Figure 1), based on the 31 cpDNA haplotypes, exhibited a sole median vector and a total of 69 mutations, 38 of which occurred along the long branch separating the Central American and Amazonian haplotype clusters. The median vector connected the single Boca do Acre haplotype 17 with the other Amazonian haplotypes. Haplotype 17 was separated by 19 mutations from the closest Central American haplotype from Panama (haplotype 7). The long branch between the Central American and Amazonian clusters is further evidence of a deep phylogeographic break.

The organization of haplotype diversity within the two geographical regions also differed. The populations from Central America exhibited a relatively low level of differentiation ( $F_{ST} = 0.36$ ) compared to Amazonian populations ( $F_{ST} = 0.91$ ) but



genetic diversity of populations was generally high ( $H_E$  ranging from 0.233 to 0.857). Some common haplotypes were shared among distantly separated Central American populations. For example, haplotypes 4 and 5 were sampled in all eight Central American populations. Haplotype 3, exhibited by 21 individuals and closely related to haplotype 4, was also widespread, occurring in four populations. These three widely distributed haplotypes represented 60% of the individuals sampled in Central America. Despite the generally weak genetic structure observed in Central American populations, haplotypes 9-16 formed a cluster comprised of individuals from the Pacific region of Panama, Costa Rica, and Guatemala, and a few individuals from a north-central Costa Rican population (El Parque).

Consistent with its higher level of population differentiation ( $F_{ST} = 0.91$ ), there were few widespread haplotypes in Amazonia. The most common and widely distributed haplotype 28 was sampled in three adjacent populations (Pimenta Bueno, Cahoeira Parecis E, Resex Chico Mendes). Four Amazonian populations were fixed for one haplotype and three other populations exhibited only two haplotypes. Haplotype diversity within populations was relatively low (mean  $H_E = 0.176$ ), with the exception of Marajoara, which contained nine haplotypes among the sixteen individuals sampled ( $H_E = 0.925$ ). One of haplotypes found in Marajoara was shared with neighboring Agua Azul, located 107 Km to the north. Populations from the western Amazon tended to cluster genetically, except for Boca do Acre, which was relatively isolated in the network and clustered with eastern rather than western populations. Haplotypes from southernmost Amazonian population (Pontes e Lacerda) tended to occupy network tips.

Spatial analysis of molecular variance (SAMOVA) indicated the most likely presence of five genetic groups ( $F_{CT} = 0.24$ ,  $P < 0.05$ ). Under all values of  $K$ , Central American populations grouped together. With  $K = 5$ , the populations grouped as

follows: 1 – Central American populations, 2 - Boca do Acre, 3 - Marajoara, Agua Azul, 4 - Pontes e Lacerda, 5 - Cachoeira Parecis A, Cachoeira Parecis E, Resex Chico Mendes, Pimenta Bueno (Figures 1 and 2). These haplotype-defined genetic groups tended to cluster the most geographically proximate populations with the exception of Resex Chico Mendes, which grouped with the populations at Cachoeira Parecis and Pimenta Bueno rather than the closer Boca do Acre.

## DISCUSSION

The cpSSR haplotype data revealed a strong phylogeographic break between *S. macrophylla* in Central America and Amazonia. Similarly large phylogeographic breaks between cis- and trans-Andean populations have been reported for other rain forest tree species (e.g. Dick et al. 2003; Dick and Heuertz 2008; Hardesty et al this issue). There were also notable differences in the distribution of cpDNA variation within Central America and Amazonia. Central American populations harboured widespread haplotypes that occurred from Mexico to Panama. The Amazonian haplotypes, on the other hand, were more localized and most cpDNA variation was partitioned among populations. This pattern is not likely to be explained by sampling effects, since the sample sizes in Brazil were consistently high (>16 individuals per population) where differentiation was also the highest.

Our results showed some inconsistency with the nuclear SSR (nSSR) analyses of Lemes et al. (2003) and Novick et al. (2003). The nSSR data from Central America showed phylogeographic structure in the form of high levels of differentiation ( $R_{ST}$ ) across geographic barriers (Novick et al., 2003). Central American populations also had relatively low allelic richness per locus (mean 13 alleles/locus) compared to the

Amazonian populations (mean of 18 alleles/locus). In contrast, there was no discernible phylogeographic structure in the Central American cpSSR data and the haplotype diversity (16 haplotypes) was similar to levels found in the Brazilian Amazonia (15 haplotypes). Some of the discrepancy between these results may be explained by differences between the nuclear and chloroplast genomes. First, genetic drift is expected to act more strongly on the chloroplast because of its fourfold lower effective population size. Furthermore, the cpDNA results reflect the sorting of a single genetic locus, whereas the nSSR results were summed over seven nSSR loci and thus provide several independent estimates of population genetic structure.

Geographic structuring of the cpSSR haplotypes does not appear to correspond with contemporary climatic or altitudinal barriers in Central America. The occurrence of widespread cpDNA haplotypes across Central America strongly implies a role of long distance dispersal and suggests that mountains have not been effective barriers to mahogany seed dispersal here. Hurricanes, which are frequent in Central America, can carry the winged seeds of mahogany over long distances and the accompanying wind throws are thought to play an important role in mahogany dispersal and establishment in this region (Snook 1996), which would lead to the present-day haplotype distribution. In the Amazon basin, on the other hand, hurricanes are absent or very rare.

On the other hand, topography may provide physical and climatic barriers for pollinator movements, as suggested by the significant divergence among Central American populations across geographical barriers found by Novick et al. (2003) using nSSR markers. Similarly, the divergences among three close populations from different valleys of the Parecis mountains in west-central Brazil, studied by Lemes et al. (2003) using nSSRs, were also highly significant, although these populations belong to the same cpSSR haplotype genetic group (group 5) in the present study.

A non-exclusive alternative explanation for to the observed phylogeographic structure in Central America is the severity of the impact of Pleistocene glaciations (Whitmore and Prance, 1987) coupled with the relatively small areas of suitable habitat for mahogany establishment. The reconstructed vegetation of lowland Central America between 20,000 and 10,500 B.P. (Piperno and Pearsall, 1998) showed restricted areas with moist and dry forests and widespread thorn woodlands, low scrub, and wooded savanna vegetation in the region. These factors are expected to have caused local extinctions and much more dramatic reduction in effective population size for Central American than for Amazonian mahogany populations (Novick et al. 2003). Under this scenario, any ancient signal of structuring and diversification would have been modified by Pleistocene vegetation changes. Thus, the current pattern of genetic variation may reflect only the most recent geographic expansion of a few founder haplotypes from a limited refugial source and the subsequent formation of newly derived haplotypes. It is worth noting the occurrence of a few rare and highly differentiated lineages (e.g. haplotypes 6 and 16) in Central America that may be a relict of the ancestral polymorphism.

The significantly lower number of nuclear microsatellite alleles and lower heterozygosity in Central American (Novick et al., 2003) than Amazonian populations (Lemes et al., 2003) suggests either a more recent geographic expansion, or lower effective population sizes in the more topographically dissected Central American region. Mating system analysis has shown that mahogany is somewhat tolerant of selfing (Lemes et al., 2007) and the lower nuclear microsatellite diversity in Central America may be influenced by ecological pressures favoring inbreeding in individuals colonizing new areas in this region.

In addition to the impacts of the Pleistocene glaciations, Holocene events may have contributed to the current phylogeographic pattern observed for mahogany in Central America. For at least 1500 years before European conquest, tens of millions of Pre-Columbian agriculturalists practiced shifting agriculture, cultivating maize and other light-demanding crops in this region (Denevan 1992). Notably, four of our sample sites (sites A-D) are in what was the most highly populated core Mayan zone of influence where several loosely associated city-states coexisted and rapid forest clearance began about 2800 B.P. (Hodell et al. 2000, Islebe et al. 1996). As a result of the intensive land use by these dense sedentary agrarian communities, Central America was probably covered, at the time of the European first arrival, by a mosaic of crop lands and abandoned fields with secondary vegetation at different successional stages. Despite the death of adult trees caused by forest clearances, this type of anthropogenic landscape will likely have enhanced dispersal of mahogany, a long-lived pioneer tree (Grogan et al. 2003), over the region. With the population decline accompanying the collapse of the Classic Mayan society between A.D. 800 and 900 (Hodell et al. 1995) and the demographic collapse experienced by Amerindian populations after European contact (Denevan 1992), human pressures were strongly curtailed and most of the fragmented landscape in Central America was abandoned. The subsequent large-scale forest regeneration (Nevle and Bird 2008) would also have accelerated the expansion of the remnant mahogany lineages in this region.

CpSSR variation in the peripheral Amazon basin exhibited a comparatively stronger phylogeographic structure than in Central America. Most of the Amazonian populations were fixed for one haplotype, or exhibited only a few related haplotypes. Spatial analysis indicates that there is significant within-region structuring in South America, primarily reflecting geographic proximity. The exception to this general

pattern is the clustering of ‘Resex Chico Mendes’ with Rondonian populations rather than the more proximate ‘Boca do Acre’. These two populations, 200 km apart and having the Juruá River as the sole geographical barrier between them, were probably derived from separate lineages. Resex Chico Mendes, located in the transitional zone between the Brazilian Shield and the Tertiary deposits of the Amazon basin was likely formed by lineages coming from the relict populations in the Serra dos Parecis mountains. Boca do Acre, located at the north-western limit of the species distribution in Brazil, seems to be more genetically related to populations from eastern Amazonia.

It seems most likely that cycles of demographic expansion followed by population bottlenecks and isolation have shaped the phylogeographic pattern in the region. Clearly, there has been a distinctly different demographic history in Amazonia compared with Central America, with an older colonization suggested by longer branch lengths and a higher level of geographic isolation for the Amazonian populations as indicated by higher  $F_{ST}$ .

The current distribution of mahogany in the Amazonia is characterized by aggregations of trees in deciduous and semi-deciduous forests along an arc following the southern boundary of the basin (Grogan, 2001; Grogan et al., 2002). These seasonally dry forests are areas of “ecological tension” between the Amazonian and Cerrado biomes and are bounded by the evergreen rain forests to the north and by savannas to the south. Seasonally dry forests, which provide optimum habitat for mahogany, probably expanded during the cool and dry glacial intervals (Pennington et al. 2000; Bush and Silman, 2004; Bush and Oliveira, 2006; Colinvaux et al. 1996, 2001) and mahogany population sizes would have been correspondingly larger and possibly more continuous in some areas. During the wetter and warmer Holocene, rainforests expanded south and eastwards replacing the deciduous forests and savannas

in the Amazonian lowlands and in the foothills of the Brazilian Shield. Thus, it is possible that the retraction and isolation of mahogany populations since the end of the Last Glacial Maximum and the lack of long distance seed dispersal among the remaining mahogany aggregations have influenced phylogeographic structure in the Brazilian Amazon.

The high level of private haplotype diversity found in the Marajoara population, in contrast to other Amazonian populations, suggests additional intricacy to the pattern. One possible explanation concerns the stability of a dry and seasonal climate in this region, even during interglacial periods. Marajoara is located on the SW border of the dry transverse corridor that crosses central Amazonia in a NW-SE direction, separating humid upper and lower Amazonia (Haffer, 2008). The private haplotype diversity of the Marajoara population may be explained by the greater size and stability of the dry forests in this area, which could have led to the accumulation and maintenance of cpSSR diversity.

The Pre-Columbian cultivation practices in Amazonia also changed the environment in different ways especially along of the headwater basins of the main rivers coming from the northern flanks of the Brazilian Shield (Heckenberger et al. 2007). This region is dominated by semi-deciduous forests and represents the natural area of distribution of *S. macrophylla* in the Brazilian and Bolivian Amazon. Several small to medium-sized complex societies flourished in this broad region with agricultural and parkland landscapes occurring around villages (Heckenberger et al. 2003, Erickson 2006). However, the lower Amerindian density, the large distances between the main settlements, the prevalence of a hunter-gatherer subsistence strategy in many groups, and the management of the landscape using agricultural systems that did not require intensive forest clearance (Denevan, 2001, Balée 2006) suggest that the

342 impact of anthropogenic disturbance in the southern Amazon during the Late Holocene  
343 was likely smaller than in Central America. One would expect that there were  
344 proportionally fewer anthropogenically-altered habitats available for mahogany  
345 colonisation in the Amazon basin than in Central America during Pre-Columbian times.  
346 Thus the present-day haplotype distribution in Amazonian mahogany appears to have  
347 been most likely shaped by Pleistocene events.

348         In addition to providing genetic evidence of regional demographic history, our  
349 study should also be useful for genetic conservation and management. In mahogany,  
350 important traits such as resistance to shoot borers, growth rate and degree of branching  
351 show heritable variation (Newton et al., 1999). The deep phylogeographic break  
352 between Central and South American mahogany populations suggests that there may be  
353 major genomic differences between these sources of mahogany. While the major  
354 provenance trials for mahogany (CATIE, Turrialba, Costa Rica) contain only Central  
355 American samples, our study strongly suggests that Central and South American  
356 provenances should be jointly studied for silviculture programs.

357         The findings also have relevance for the conservation of natural mahogany  
358 populations. Recent advances in DNA extraction technology permit genotyping of DNA  
359 from dried timber samples (Deguilloux et al., 2002). Using a DNA barcoding approach,  
360 it is possible to determine the species origin of tropical timbers. However, standard  
361 plant DNA barcodes often display little variation among closely related species, let  
362 alone between populations within a species (Dick and Kress, 2009), making it difficult  
363 to determine the provenance of timber, which is essential in order to monitor illegal  
364 logging activities. Our study demonstrates that combinations of cpSSR loci can provide  
365 distinct regional cpDNA haplotypes for mahogany. With the six cpSSR loci in this  
366 population, it was possible to definitively assign samples to either Central American or



South American provenances. With more loci, it should be possible to provide distinct genotypes at finer geographic scales. These DNA barcode approaches should be especially useful in the Amazon basin, which displays the highest level of cpDNA phylogeographic structure, and which contains the largest commercial and protected tracts of mahogany.

In summary, our data have highlighted a strong phylogeographic break and an intriguing contrast between Amazonian and Central American mahogany populations in terms of phylogeographic structure. In order to clarify the occurrence of points of historical dispersal between the two geographical regions, more extensive sampling in South America is needed, particularly from the Peruvian, Ecuadorian, Colombian and Venezuelan Amazon. Based on the data available so far we suggest that differences in glacial history for Central America and Amazonia may have been a key factor in determining these very divergent patterns. Differences in terms of duration and intensity of anthropogenic disturbance between the two regions during the late Holocene may have also affected the vegetation history and played an important role in structuring cpDNA lineages. In addition, these findings indicate that any *in situ* conservation program or germplasm collection initiatives for this valuable and endangered tree should take into consideration the distinct genetic structures shaped by the contrasting history of the species in these regions.

## **METHODS**

### **Study sites and collection**

Leaves of 245 plants were collected from eight populations from six countries in Central America and from eight populations spread across 2,100 km in the southern arc of the Amazon drainage basin in Brazil (Figure 2). The samples were used in previous

nuclear microsatellite analyses of Lemes et al. (2003) and Novick et al. (2003). In addition, new leaf material from adult trees was sampled from Boca do Acre in Brazil (N = 28). The leaf material came from adult trees from natural populations in Amazonia except for Cach E and Agua Azul or from progeny arrays for the Central American populations and Cach E and Agua Azul, in which a single progeny was used as a proxy for an adult tree. Living material from the Central American populations is maintained by CATIE in Turrialba, Costa Rica. The leaves were dried in silica gel and stored at -20°C until DNA extraction.

#### **Microsatellite analysis**

Total genomic DNA was extracted using a Fast Prep (Bio101 Corporation) following standard CTAB procedure (Doyle and Doyle, 1987) or alternatively using Plant DNeasy kits (Qiagen Corporation, Valencia, CA). DNA quantification was performed by comparison with known concentrations of a DNA standard (Lambda DNA) in ethidium bromide-stained 1% agarose gels.

PCR was initially performed using 10 universal angiosperm primers developed by Weising & Gardner (1999) for cpSSR analysis in tobacco. Reactions were carried out in a total volume of 10 µl containing 1X PCR buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM MgCl<sub>2</sub>), 200 µM dNTPs, BSA (2.5 mg ml<sup>-1</sup>), 1.25 µM of each forward and reverse primers, 1 U Taq DNA polymerase and 5.0 ng of genomic DNA using a MJ Research Incorporated PTC 200 thermal cycler under the following parameters: (1) initial denaturation at 94°C for 4 min; (2) 30 cycles of denaturation at 94°C for 1 min, annealing at primer-specific  $T_m$  for 1 min and extension at 72°C for 1 min; (3) final extension at 72°C for 10 min. PCR products were eletrophoresed on 5% polyacrylamide gels in an Applied Biosystem Incorporated (ABI) Prism 377 sequencer

and analysed with Genescan and Genotyper softwares (ABI). The cpSSR allele sizes were binned and normalized using *AlleloBin* software (Idury and Cardon, 1997).

## **Data analysis**

Unique multi-locus combinations of cpSSR alleles (size variants) were considered as distinct haplotypes. Genetic diversity was estimated for each population based on the number of alleles ( $A$ ), the number of haplotypes ( $N_H$ ), and gene diversity index ( $H_E$ , Nei, 1987). Partitioning of genetic variation within and among populations was tested for each geographical region (Amazonia and Central America) separately by analysis of molecular variation (AMOVA; Excoffier et al., 1992) using *Arlequin 2.001* (Schneider et al., 2001). The significance of the fixation index was tested with 1000 permutations. Relationships among the haplotypes were inferred using median-joining network analysis (Bandelt et al., 1999) implemented by *Network* software (Forster et al., 2000). Spatial structuring of variation at chloroplast loci was examined using SAMOVA (Dupanloup et al., 2002), considering values of  $K$  (phylogroup number) between 2 and 10, using 100 initial conditions for each run and the sum of squared size differences as a measure of molecular distance.

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