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Contact CEH NORA team at
noraceh@ceh.ac.uk

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5   Chloroplast DNA microsatellites reveal contrasting phylogeographic structure in

6   mahogany (*Swietenia macrophylla* King, Meliaceae) from Amazonia and Central

7   America.

8

9   MARISTERRA R. LEMES[1*], CHRISTOPHER W. DICK[2,3], CARLOS NAVARRO[4,5],

10   ANDREW J. LOWE[6], STEPHEN CAVERS[7] AND ROGÉRIO GRIBEL[1]

11

12   [1]Instituto Nacional de Pesquisas da Amazônia, Laboratório de Genética e Biologia

13   Reprodutiva de Plantas, Av. André Araújo, 2936, 69083-000 Manaus-AM, Brazil.

14

15   [2]University of Michigan, Department of Ecology and Evolutionary Biology and

16   University Herbarium, Ann Arbor, MI 48109 USA

17

18   [3]Smithsonian Tropical Research Institute, P.O. Box 0843-03092, Balboa, Ancón,

19   Republic of Panama

20

21   [4]Centro Agronómico Tropical de Investigación & Ensenanza, Cartago, Turrialba 7170,

22   Costa Rica

23

24   [5]Universidad Nacional, Instituto de Investigaciones y Servicios Forestales (INISEFOR),

25   86-3000 Heredia, Costa Rica.

26

27   [6]Australian Centre for Evolutionary Biology and Biodiversity, School of Earth and

28   Environmental Sciences, Univeristy of Adelaide, Adelaide, SA 5000, Australia and

29   State Herbarium, Science Resource Centre, Department for Environment and Heritage,

30   Adelaide, SA 5005, Australia

31

32   [7]NERC Centre for Ecology and Hydrology, Bush Estate, Penicuik, Midlothian,

33   Scotland. EH26 0QB

34

35   [*]Correspondence: Maristerra R. Lemes, Instituto Nacional de Pesquisas da Amazônia,

36   Laboratório de Genética e Biologia Reprodutiva de Plantas, Av. André Araújo, 2936,

37   69083-000 Manaus-AM, Brazil, Fax number: + 55 92 36433285, email:

38   mlemes@inpa.gov.br

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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.

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. S*wietenia 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 20th 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;

4

93   Gullison et al., 1996). In addition to removing the most fecund trees, selective logging

94   may have a significant impact on genetic structure and population size, and compromise

95   the evolutionary viability of natural mahogany populations (Cornelius et al., 2005). The

96   inclusion of *S. macrophylla* in CITES Appendix II in 2002 aimed to control

97   international trade by ensuring that logging will not be detrimental to the survival of the

98   species (Grogan and Barreto, 2005).

99   Studies of the organization of genetic diversity of *S. macrophylla* have been

100 carried out in the Brazilian Amazonia (Lemes et. al., 2003) and in Central America

101 (Novick et al. 2003) using nuclear microsatellite DNA markers. The studies sampled

102 populations across a similar spatial scale (ca. 1600 km in Central America; 2103 km in

103 Amazonia) using seven loci in common. Both studies showed significant isolation by

104 distance patterns, and moderate levels of population differentiation (R_{ST}). Furthermore,

105 the Central American populations exhibited significantly lower mean genetic diversity

106 than the Amazonian populations, which Novick et al. (2003) suggested may have

107 resulted from the smaller, more dissected nature of suitable habitat in Central America,

108 combined with more severe vegetation changes during the glacial phases of the

109 Pleistocene.

110   A phylogeographic approach based on chloroplast genome (cpDNA) variation

111 can provide additional insight into the historical patterns of genetic divergence across

112 the range of *S. macrophylla*. Chloroplast DNA is a haploid genome and is maternally

113 inherited in the majority of the angiosperms (Birky, 1995; McCauley 1995; Ennos et al

114 1999). Because of its four-fold smaller effective population size, chloroplast markers

115 can often detect geographic structure that is not apparent in nuclear DNA markers

116 (Cavers et al., 2003; Petit, 2005). Unfortunately the relatively low rates of nucleotide

117 substitution in the chloroplast genome (Wolfe et al., 1987) have often impeded its use in

118   phylogeographic studies (Schaal, 1998). However, highly variable mononucleotide

119   repeat loci in the chloroplast genome (cpDNA microsatellites or simple sequence

120   repeats [cpSSRs]) have provided a rich source of variation for studies of

121   phylogeography and gene flow (Provan et al., 2001).

122   In addition to its utility for phylogeographic studies, conservation and

123   management purposes, cpSSRs may be useful as regionally distinct cpDNA barcodes

124   that could permit forensic verification of timber origins (Deguilloux et al., 2002; Dick

125   and Kress, 2009). Here we report on the phylogeographic structure of *S. macrophylla*

126   populations sampled in Central America (Novick et al. 2003) and Amazonia (Lemes et

127   al. 2003) based on cpDNA microsatellites. The main aims of the study were: (1) to

128   evaluate the utility of chloroplast microsatellite loci for assessing intraspecific variation

129   in *S. macrophylla*; (2) to quantify and compare the organization of genetic diversity of

130   *S. macrophylla* populations in Central America and Amazonia ; and (3) to determine if

131   population-specific cpDNA haplotypes are credible as regional DNA barcodes for

132   monitoring timber harvests.

133

134   **RESULTS**

135   **Genetic Variation**

136   Six out of 10 cpSSR loci initially assayed using universal primers (Weising and

137   Gardner, 1999) successfully amplified and were found to be polymorphic. All

138   individuals (n = 245) from eight Central American and eight Amazonian populations

139   were analyzed for these six polymorphic cpSSR loci (ccmp 2, ccmp 3, ccmp 4, ccmp 5,

140   ccmp 7, ccmp 10).

141   A total of 31 different haplotypes and 30 cpSSR alleles were found. The

142   composition of the haplotypes and their distribution in the populations are given in

143   Table 1. The number of size variants (alleles) per locus varied from three to six. Gene

144   diversity indices (H_E) showed a high range of variation across populations (0.000 to

145   0.925) (Table 2).

146

147   **Structuring and geographical distribution of haplotypes**

148   The hierarchical analysis of genetic variation within and among populations performed

149   for each geographical region (Amazonia and Central America) showed contrasting

150   patterns. Most of the variation found in Amazonia was partitioned among populations

151   (91%), while in Central America most variation was partitioned within populations

152   (64%, Table 3).

153   The pattern of cpSSR haplotype organization provides evidence of a strong

154   phylogeographic break between *S. macrophylla* populations in Central America and

155   Amazonia. Of the 31 haplotypes detected, 15 occurred exclusively in Amazonian

156   populations and the remaining 16 in Central America (Table 2), with no single

157   haplotype shared between the two geographical regions. A maximum parsimony

158   median-joining network (Figure 1), based on the 31 cpDNA haplotypes, exhibited a sole

159   median vector and a total of 69 mutations, 38 of which occurred along the long branch

160   separating the Central American and Amazonian haplotype clusters. The median vector

161   connected the single Boca do Acre haplotype 17 with the other Amazonian haplotypes.

162   Haplotype 17 was separated by 19 mutations from the closest Central American

163   haplotype from Panama (haplotype 7). The long branch between the Central American

164   and Amazonian clusters is further evidence of a deep phylogeographic break.

165   The organization of haplotype diversity within the two geographical regions also

166   differed. The populations from Central America exhibited a relatively low level of

167   differentiation ($F_{ST} = 0.36$) compared to Amazonian populations ($F_{ST} = 0.91$) but

168   genetic diversity of populations was generally high (H_E ranging from 0.233 to 0.857).

169   Some common haplotypes were shared among distantly separated Central American

170   populations. For example, haplotypes 4 and 5 were sampled in all eight Central

171   American populations. Haplotype 3, exhibited by 21 individuals and closely related to

172   haplotype 4, was also widespread, occurring in four populations. These three widely

173   distributed haplotypes represented 60% of the individuals sampled in Central America.

174   Despite the generally weak genetic structure observed in Central American populations,

175   haplotypes 9-16 formed a cluster comprised of individuals from the Pacific region of

176   Panama, Costa Rica, and Guatemala, and a few individuals from a north-central Costa

177   Rican population (El Parque).

178   Consistent with its higher level of population differentiation (F_{ST} = 0.91), there

179   were few widespread haplotypes in Amazonia. The most common and widely

180   distributed haplotype 28 was sampled in three adjacent populations (Pimenta Bueno,

181   Cahoeira Parecis E, Resex Chico Mendes). Four Amazonian populations were fixed for

182   one haplotype and three other populations exhibited only two haplotypes. Haplotype

183   diversity within populations was relatively low (mean H_E = 0.176), with the exception

184   of Marajoara, which contained nine haplotypes among the sixteen individuals sampled

185   *(H_E = 0.925).* One of haplotypes found in Marajoara was shared with neighboring Agua

186   Azul, located 107 Km to the north. Populations from the western Amazon tended to

187   cluster genetically, except for Boca do Acre, which was relatively isolated in the

188   network and clustered with eastern rather than western populations. Haplotypes from

189   southernmost Amazonian population (Pontes e Lacerda) tended to occupy network tips.

190   Spatial analysis of molecular variance (SAMOVA) indicated the most likely

191   presence of five genetic groups (F_{CT} = 0.24, P<0.05). Under all values of K, Central

192   American populations grouped together. With K = 5, the populations grouped as

193   follows: 1 – Central American populations, 2 - Boca do Acre, 3 - Marajoara, Agua

194   Azul, 4 - Pontes e Lacerda, 5 - Cachoeira Parecis A, Cachoeira Parecis E, Resex Chico

195   Mendes, Pimenta Bueno (Figures 1 and 2). These haplotype-defined genetic groups

196   tended to cluster the most geographically proximate populations with the exception of

197   Resex Chico Mendes, which grouped with the populations at Cachoeira Parecis and

198   Pimenta Bueno rather than the closer Boca do Acre.

199

200   **DISCUSSION**

201

202   The cpSSR haplotype data revealed a strong phylogeographic break between *S.*

203   *macrophylla* in Central America and Amazonia. Similarly large phylogeographic breaks

204   between cis- and trans-Andean populations have been reported for other rain forest tree

205   species (e.g. Dick et al. 2003; Dick and Heuertz 2008; Hardesty et al this issue). There

206   were also notable differences in the distribution of cpDNA variation within Central

207   America and Amazonia. Central American populations harboured widespread

208   haplotypes that occurred from Mexico to Panama. The Amazonian haplotypes, on the

209   other hand, were more localized and most cpDNA variation was partitioned among

210   populations. This pattern is not likely to be explained by sampling effects, since the

211   sample sizes in Brazil were consistently high (>16 individuals per population) where

212   differentiation was also the highest.

213   Our results showed some inconsistency with the nuclear SSR (nSSR) analyses of

214   Lemes et al. (2003) and Novick et al. (2003). The nSSR data from Central America

215   showed phylogeographic structure in the form of high levels of differentiation (R_{ST})

216   across geographic barriers (Novick et al., 2003). Central American populations also had

217   relatively low allelic richness per locus (mean 13 alleles/locus) compared to the

218   Amazonian populations (mean of 18 alleles/locus). In contrast, there was no discernible

219   phylogeographic structure in the Central American cpSSR data and the haplotype

220   diversity (16 haplotypes) was similar to levels found in the Brazilian Amazonia (15

221   haplotypes). Some of the discrepancy between these results may be explained by

222   differences between the nuclear and chloroplast genomes. First, genetic drift is expected

223   to act more strongly on the chloroplast because of its fourfold lower effective

224   population size. Furthermore, the cpDNA results reflect the sorting of a single genetic

225   locus, whereas the nSSR results were summed over seven nSSR loci and thus provide

226   several independent estimates of population genetic structure.

227   Geographic structuring of the cpSSR haplotypes does not appear to correspond

228   with contemporary climatic or altitudinal barriers in Central America. The occurrence of

229   widespread cpDNA haplotypes across Central America strongly implies a role of long

230   distance dispersal and suggests that mountains have not been effective barriers to

231   mahogany seed dispersal here. Hurricanes, which are frequent in Central America, can

232   carry the winged seeds of mahogany over long distances and the accompanying wind

233   throws are thought to play an important role in mahogany dispersal and establishment in

234   this region (Snook 1996), which would lead to the present-day haplotype distribution. In

235   the Amazon basin, on the other hand, hurricanes are absent or very rare.

236   On the other hand, topography may provide physical and climatic barriers for

237   pollinator movements, as suggested by the significant divergence among Central

238   American populations across geographical barriers found by Novick et al. (2003) using

239   nSSR markers. Similarly, the divergences among three close populations from different

240   valleys of the Parecis mountains in west-central Brazil, studied by Lemes et al. (2003)

241   using nSSRs, were also highly significant, although these populations belong to the

242   same cpSSR haplotype genetic group (group 5) in the present study.

243   A non-exclusive alternative explanation for to the observed phylogeographic

244   structure in Central America is the severity of the impact of Pleistocene glaciations

245   (Whitmore and Prance, 1987) coupled with the relatively small areas of suitable habitat

246   for mahogany establishment. The reconstructed vegetation of lowland Central America

247   between 20,000 and 10,500 B.P. (Piperno and Pearsall, 1998) showed restricted areas

248   with moist and dry forests and widespread thorn woodlands, low scrub, and wooded

249   savanna vegetation in the region. These factors are expected to have caused local

250   extinctions and much more dramatic reduction in effective population size for Central

251   American than for Amazonian mahogany populations (Novick et al. 2003). Under this

252   scenario, any ancient signal of structuring and diversification would have been modified

253   by Pleistocene vegetation changes. Thus, the current pattern of genetic variation may

254   reflect only the most recent geographic expansion of a few founder haplotypes from a

255   limited refugial source and the subsequent formation of newly derived haplotypes. It is

256   worth noting the occurrence of a few rare and highly differentiated lineages (e.g.

257   haplotypes 6 and 16) in Central America that may be a relict of the ancestral

258   polymorphism.

259   The significantly lower number of nuclear microsatellite alleles and lower

260   heterozygosity in Central American (Novick et al., 2003) than Amazonian populations

261   (Lemes et al., 2003) suggests either a more recent geographic expansion, or lower

262   effective population sizes in the more topographically dissected Central American

263   region. Mating system analysis has shown that mahogany is somewhat tolerant of

264   selfing (Lemes et al., 2007) and the lower nuclear microsatellite diversity in Central

265   America may be influenced by ecological pressures favoring inbreeding in individuals

266   colonizing new areas in this region.

267   In addition to the impacts of the Pleistocene glaciations, Holocene events may

268   have contributed to the current phylogeographic pattern observed for mahogany in

269   Central America. For at least 1500 years before European conquest, tens of millions of

270   Pre-Columbian agriculturalists practiced shifting agriculture, cultivating maize and

271   other light-demanding crops in this region (Denevan 1992). Notably, four of our sample

272   sites (sites A-D) are in what was the most highly populated core Mayan zone of

273   influence where several loosely associated city-states coexisted and rapid forest

274   clearance began about 2800 B.P. (Hodell et al.2000, Islebe et al. 1996). As a result of

275   the intensive land use by these dense sedentary agrarian communities, Central America

276   was probably covered, at the time of the European first arrival, by a mosaic of crop

277   lands and abandoned fields with secondary vegetation at different successional stages.

278   Despite the death of adult trees caused by forest clearances, this type of anthropogenic

279   landscape will likely have enhanced dispersal of mahogany, a long-lived pioneer tree

280   (Grogan et al. 2003), over the region. With the population decline accompanying the

281   collapse of the Classic Mayan society between A.D. 800 and 900 (Hodell et al. 1995)

282   and the demographic collapse experienced by Amerindian populations after European

283   contact (Denevan 1992), human pressures were strongly curtailed and most of the

284   fragmented landscape in Central America was abandoned . The subsequent large-scale

285   forest regeneration (Nevle and Bird 2008) would also have accelerated the expansion of

286   the remnant mahogany lineages in this region.

287   CpSSR variation in the peripheral Amazon basin exhibited a comparatively

288   stronger phylogeographic structure than in Central America. Most of the Amazonian

289   populations were fixed for one haplotype, or exhibited only a few related haplotypes.

290   Spatial analysis indicates that there is significant within-region structuring in South

291   America, primarily reflecting geographic proximity. The exception to this general

292 pattern is the clustering of 'Resex Chico Mendes' with Rondonian populations rather

293 than the more proximate 'Boca do Acre'. These two populations, 200 km apart and

294 having the Juruá River as the sole geographical barrier between them, were probably

295 derived from separate lineages. Resex Chico Mendes, located in the transitional zone

296 between the Brazilian Shield and the Tertiary deposits of the Amazon basin was likely

297 formed by lineages coming from the relict populations in the Serra dos Parecis

298 mountains. Boca do Acre, located at the north-western limit of the species distribution

299 in Brazil, seems to be more genetically related to populations from eastern Amazonia.

300   It seems most likely that cycles of demographic expansion followed by

301 population bottlenecks and isolation have shaped the phylogeographic pattern in the

302 region. Clearly, there has been a distinctly different demographic history in Amazonia

303 compared with Central America, with an older colonization suggested by longer branch

304 lengths and a higher level of geographic isolation for the Amazonian populations as

305 indicated by higher F_{ST}.

306   The current distribution of mahogany in the Amazonia is characterized by

307 aggregations of trees in deciduous and semi-deciduous forests along an arc following

308 the southern boundary of the basin (Grogan, 2001; Grogan et al., 2002). These

309 seasonally dry forests are areas of "ecological tension" between the Amazonian and

310 Cerrado biomes and are bounded by the evergreen rain forests to the north and by

311 savannas to the south. Seasonally dry forests, which provide optimum habitat for

312 mahogany, probably expanded during the cool and dry glacial intervals (Pennington et

313 al. 2000; Bush and Silman, 2004; Bush and Oliveira, 2006; Colinvaux et al. 1996,

314 2001) and mahogany population sizes would have been correspondingly larger and

315 possibly more continuous in some areas. During the wetter and warmer Holocene,

316 rainforests expanded south and eastwards replacing the deciduous forests and savannas

317   in the Amazonian lowlands and in the foothills of the Brazilian Shield. Thus, it is

318   possible that the retraction and isolation of mahogany populations since the end of the

319   Last Glacial Maximum and the lack of long distance seed dispersal among the

320   remaining mahogany aggregations have influenced phylogeographic structure in the

321   Brazilian Amazon.

322   The high level of private haplotype diversity found in the Marajoara population,

323   in contrast to other Amazonian populations, suggests additional intricacy to the pattern.

324   One possible explanation concerns the stability of a dry and seasonal climate in this

325   region, even during interglacial periods. Marajoara is located on the SW border of the

326   dry transverse corridor that crosses central Amazonia in a NW-SE direction, separating

327   humid upper and lower Amazonia (Haffer, 2008). The private haplotype diversity of the

328   Marajoara population may be explained by the greater size and stability of the dry

329   forests in this area, which could have led to the accumulation and maintenance of

330   cpSSR diversity.

331   The Pre-Columbian cultivation practices in Amazonia also changed the

332   environment in different ways especially along of the headwater basins of the main

333   rivers coming from the northern flanks of the Brazilian Shield (Heckenberger et al.

334   2007). This region is dominated by semi-deciduous forests and represents the natural

335   area of distribution of *S. macrophylla* in the Brazilian and Bolivian Amazon. Several

336   small to medium-sized complex societies flourished in this broad region with

337   agricultural and parkland landscapes occurring around villages (Heckenberger et al.

338   2003, Erickson 2006). However, the lower Amerindian density, the large distances

339   between the main settlements, the prevalence of a hunter-gatherer subsistence strategy

340   in many groups, and the management of the landscape using agricultural systems that

341   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

367 South American provenances. With more loci, it should be possible to provide distinct

368 genotypes at finer geographic scales. These DNA barcode approaches should be

369 especially useful in the Amazon basin, which displays the highest level of cpDNA

370 phylogeographic structure, and which contains the largest commercial and protected

371 tracts of mahogany.

372 In summary, our data have highlighted a strong phylogeographic break and an

373 intriguing contrast between Amazonian and Central American mahogany populations in

374 terms of phylogeographic structure. In order to clarify the occurrence of points of

375 historical dispersal between the two geographical regions, more extensive sampling in

376 South America is needed, particularly from the Peruvian, Ecuadorian, Colombian and

377 Venezuelan Amazon. Based on the data available so far we suggest that differences in

378 glacial history for Central America and Amazonia may have been a key factor in

379 determining these very divergent patterns. Differences in terms of duration and intensity

380 of anthropogenic disturbance between the two regions during the late Holocene may

381 have also affected the vegetation history and played an important role in structuring

382 cpDNA lineages. In addition, these findings indicate that any *in situ* conservation

383 program or germplasm collection initiatives for this valuable and endangered tree

384 should take into consideration the distinct genetic structures shaped by the contrasting

385 history of the species in these regions.

386

387 **METHODS**

388 **Study sites and collection**

389 Leaves of 245 plants were collected from eight populations from six countries in

390 Central America and from eight populations spread across 2,100 km in the southern arc

391 of the Amazon drainage basin in Brazil (Figure 2). The samples were used in previous

392   nuclear microsatellite analyses of Lemes et al. (2003) and Novick et al. (2003). In

393   addition, new leaf material from adult trees was sampled from Boca do Acre in Brazil

394   (N = 28). The leaf material came from adult trees from natural populations in Amazonia

395   except for Cach E and Agua Azul or from progeny arrays for the Central American

396   populations and Cach E and Agua Azul, in which a single progeny was used as a proxy

397   for an adult tree. Living material from the Central American populations is maintained

398   by CATIE in Turrialba, Costa Rica. The leaves were dried in silica gel and stored at -

399   20°C until DNA extraction.

400

401   **Microsatellite analysis**

402   Total genomic DNA was extracted using a Fast Prep (Bio101 Corporation)

403   following standard CTAB procedure (Doyle and Doyle, 1987) or alternatively using

404   Plant DNeasy kits (Qiagen Corporation, Valencia, CA). DNA quantification was

405   performed by comparison with known concentrations of a DNA standard (Lambda

406   DNA) in ethidium bromide-stained 1% agarose gels.

407   PCR was initially performed using 10 universal angiosperm primers developed

408   by Weising & Gardner (1999) for cpSSR analysis in tobacco. Reactions were carried

409   out in a total volume of 10 µl containing 1X PCR buffer (10 mM Tris-HCl, pH 8.3, 50

410   mM KCl, 1.5 mM $MgCl_2$), 200 µM dNTPs, BSA (2.5 mg ml^{-1}), 1.25 µM of each

411   forward and reverse primers, 1 U Taq DNA polymerase and 5.0 ng of genomic DNA

412   using a MJ Research Incorporated PTC 200 thermal cycler under the following

413   parameters: (1) initial denaturation at 94^{0}C for 4 min; (2) 30 cycles of denaturation at

414   94^{0}C for 1 min, annealing at primer-specific T_m for 1 min and extension at 72^{0}C for 1

415   min; (3) final extension at 72^{0}C for 10 min. PCR products were eletrophoresed on 5%

416   polyacrylamide gels in an Applied Biosystem Incorporated (ABI) Prism 377 sequencer

417 and analysed with Genescan and Genotyper softwares (ABI). The cpSSR allele sizes

418 were binned and normalized using *AlleloBin* software (Idury and Cardon, 1997).

419

420 **Data analysis**

421   Unique multi-locus combinations of cpSSR alleles (size variants) were

422 considered as distinct haplotypes. Genetic diversity was estimated for each population

423 based on the number of alleles (A), the number of haplotypes (N_H), and gene diversity

424 index (H_E, Nei, 1987). Partitioning of genetic variation within and among populations

425 was tested for each geographical region (Amazonia and Central America) separately by

426 analysis of molecular variation (AMOVA; Excoffier et al., 1992) using *Arlequin 2.001*

427 (Schneider et al., 2001). The significance of the fixation index was tested with 1000

428 permutations. Relationships among the haplotypes were inferred using median-joining

429 network analysis (Bandelt et al., 1999) implemented by *Network* software (Forster et al.,

430 2000). Spatial structuring of variation at chloroplast loci was examined using

431 SAMOVA (Dupanloup et al, 2002), considering values of K (phylogroup number)

432 between 2 and 10, using 100 initial conditions for each run and the sum of squared size

433 differences as a measure of molecular distance.

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