Chloroplast DNA Variation of *Carapa guianensis* in the Amazon basin

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Abstract

Carapa guianensis is a widespread Neotropical tree species that produces a seed adapted for water dispersal. We conducted a pilot study of chloroplast DNA (cpDNA) variation in order to investigate the consequences of hydrochory on genetic diversity and geographic population structure in the lower Amazon basin. A survey of cpDNA haplotype variation reveals a strong regional structure, which suggests limited gene flow by seeds. Within site variation was detected only in one floodplain forest (*varzea*), suggesting that seed dispersal by water in these forests has the potential to mix maternal lineages. Several phylogeographic hypotheses are discussed with respect to these data.

Key words: cpDNA, Amazonia, Amazon River, *Carapa guianensis*, seed dispersal.

Introduction

An understanding of gene dispersal can help account for patterns and levels of biodiversity. In plant populations, genes disperse through the movement of pollen and seeds. Seed dispersal, and in particular, long-distance seed dispersal, allows for migration and colonization of new areas, and contributes to the genetic structure of populations.

Chloroplast DNA (cpDNA) is usually maternally inherited in angiosperms, and is thereby dispersed by seed. This allows one to use patterns of variation in cpDNA to help infer historical processes of seed migration and colonization. Since cpDNA evolves slowly, the observed patterns of cpDNA variation are likely to arise from processes occurring over long time periods and large spatial scales. CpDNA has emerged as the marker of choice for examining patterns of gene flow through seed dispersal. An especially interesting set of examples is the study of Pleistocene tree colonization in Europe (Petit *et al.,* 2003).

The few studies using cpDNA that have been conducted in the tropics suggest that spatiotemporal patterns of cpDNA variation are the product of both biogeographical history and contemporary seed dispersal, but the imprint of Pleistocene recolonization events in genetic diversity may be more diffuse and difficult to detect in the tropics compared with temperate zones (Caron *et al.,* 2000; Dutech *et al.,* 2000; Cavers *et al.,* 2003). Despite long-standing interest in the origin and history of biodiversity in the Amazon basin, and the potential role of the Amazon River system as a conduit of dispersal, to date there have been no published studies using cpDNA to infer phylogeographic patterns there.

Hydrochory (seed dispersal by water) is an important means of seed dispersal in several types of rainforests that flood seasonally or tidally. Near the ocean, seed from forests subjected to tidal flooding may be dispersed both upstream or downstream. Fish could also play a role in dispersing seed, and this could further contribute to upstream dispersal in seasonally inundated forests (Gottsberger, 1978). In areas where seed are dispersed by water, there is the potential for extensive genetic exchange between populations in the Amazon basin. Therefore we expect cpDNA variation to be weakly structured at this scale. We would also expect to find a higher level of variation at downstream flooded sites located near the Amazon River mouth compared with upstream sites.

This study was initiated in an attempt to assess the consequences of hydrochory in shaping the genetic diversity of the Neotropical forest tree, *Carapa guianensis* (Meliaceae). We describe cpDNA variation for this species as revealed by polymerase chain reaction (PCR) of chloroplast intergenic sequences, followed by analysis of restriction fragment length polymorphisms (RFLPs), and we use the variation observed to examine hypotheses pertaining to seed dispersal in this species. Specifically, we examine: (1) whether genetic differentiation among sites is consistent with the presence of hydrochory in the Amazon basin; and (2) whether there is evidence that flooded forests receive seed migrants from distant sites.

Materials and Methods

Study species

Carapa guianensis Aubl. (Meliaceae) is a widespread Neotropical tree found in the Caribbean islands and Central America to northern South America, including Amazonia. The species inhabits upland *terra firme* forests as well as floodplain *varzea* forests in the Amazon basin. A second species in the genus, *C. procera*, is also found in the New World. This species is restricted to the Guyanas and Central Amazonia, but is also found in Africa. The two species can be differentiated from one another by reproductive morphology (Pennington, 1981; Dendrogene, 2004), but it is difficult to distinguish

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trees and saplings in the field when flowers or fruits are absent. At the seedling stage, *C. guianensis* has composite leaves, while *C. procera* has simple leaves (FISCH *et al.,* 1995).

The seed of *Carapa guianensis* is composed of a large kernel, variable in weight (10–50 g), and containing a high proportion of lipids. The seeds and seedlings are typically found directly beneath the parent tree, and occasionally are dispersed a few meters by scatter-hoarding rodents (FORGET *et al.,* 1999). Along streams and in areas subjected to flooding, however, the seed may float and be carried by water currents. They are often found in large numbers on the riverbanks at time of seed release. The seed of this species are adapted for water dispersal and can easily germinate and establish after a floating phase of several months (SCARANO *et al.,* 2003). *C. guianensis* is also used by the local human population as source of timber and medicinal oil, which is extracted from the seed.

Sampling

Due to legal restrictions on sampling *Carapa guianensis* material for genetic analyses, we used material held by the EMBRAPA (Empresa Brasileira de Pesquisa Agropecuaria) research network, together with seedlings sold by local merchants. Twenty-two dried samples from the Ian herbarium of EMBRAPA-Bélem and one sample from the Cen herbarium of EMBRAPA-Brasilia were collected. These samples represent diverse locations from Brazil, Venezuela and Suriname, and collection dates ranging from 1942 to 2002. The fresh samples were collected from EMBRAPA research sites, or bought from people involved in the trade of seed and seedling of tree species (*Table 1*). These samples were dried in silica gel before being sent to the lab.

Laboratory analyses

For the herbarium specimens, DNA was extracted using the DNeasy plant mini-kit (Quiagen) following the standard protocol with slight modifications as described in DRABKOVA *et al.* (2002). For the fresh samples, DNA was extracted from about 200 mg of leaf tissue using a standard CTAB protocol. Chloroplast DNA intergenic sequences were amplified using a set of 16 universal primer pairs developed by TABERLET *et al.* (1991), DEMESURE *et al.* (1995) and DUMOLIN-LAPEGUE *et al.* (1997). The PCR reaction mixture was in a final volume of 13 µL containing: DNA (0.3 ng/µL), reaction buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$), primers (0.5 µM each), $MgCl_2$ (1.5 mM), BSA (0.25 mg/mL), dNTP (0.25 mM) and *Taq* polymerase (1.3 U, Invitrogen). PCR amplifications were carried out using 1 cycle of 4 min 94 °C, 30 cycles of 45 s at 92 °C, 45 s at 54 °C, 3 min at 72 °C and one cycle of 10 min at 72 °C. PCR products were digested using various restriction enzymes, following manufacturer's specifications. Restriction fragments were then separated in 3% agarose gels stained with ethidium bromide and visualized by UV fluorescence. Several digital pictures were taken during migration to maximize the chances of detecting variants.

To allow an initial screen of geographical variation a subset of chloroplast amplification products from Acre, Belterra, Porto Trombetas and Belem were digested with four different restriction enzymes (*Hinf*I, *Taq*1, *Mse*I, *Alu*I). Those enzyme-primer pair combinations that revealed variation among locations were used for analysis of the remaining samples. When several different enzymes revealed variation for a given primer pair, the enzyme-primer pair combination that gave the clearest variable bands under migration conditions used was

Table 1. – Carapa guianensis populations analyzed for chloroplast DNA variation.

Location	Number* and name of location	Source of material	Species	Sample size	Haplotype
00°05'N 51°08'W	1.Macapa AP	Terra firme forest	*C. guianensis/procera*	1	A
01°00'S 51°30'W	2.Gurupa Island PA	Varzea forest	*C. guianensis*	9	A(8), B(1)
01°10'S 48°20'W	3.Santa Barbara PA	Terra firme forest	*C. guianensis*	1	A
01°15'S 56°38'W	4.Porto Trombetas PA	Tree nursery	*C. guianensis*†	3	C
01°20'S 51°38'W	5.Gurupa Town PA	Seedlings on riverbanks	*C. guianensis*†	6	A
01°25'S 53°48'W	6.Prainha PA - Jatuarana	Terra firme forest	*C. guianensis*	1	A
01°30'S 48°30'W	7.Belem PA - Embrapa	Plantation	*C. guianensis*	1	A
01°34'S 56°15'W	8.Oriximina PA - Trombetas river	Plantation	*C. guianensis/procera*	1	D
01°40'S 55°52'W	9.Oriximina PA - Town	Tree nursery	*C. procera*†	3	C
01°54'S 52°27'W	10.Porto de Moz PA - Arurua	Plantation	*C. guianensis*	1	A
02°31'S 54°39'W	11.Santarem PA - Curua-Una road	Terra firme forest	*C. guianensis*	1	E
02°32'S 54°07'W	12.Santarem PA - Curua-Una	Plantation	*C. guianensis*	1	E
02°50'S 52°01'W	13.Vitoria do Xingu PA - Town	Plantation	*C. guianensis*	1	A
02°50'S 55°01'W	14.Belterra PA - Flona km64	Terra firme forest	*C. guianensis*	2	E
03°01'S 54°58'W	15.Belterra PA - Flona km83	Terra firme forest	*C. guianensis*	3	E
06°13'S 57°55'W	16.Jacareacanga PA	Terra firme forest	*C. guianensis/procera*	1	E
09°45'S 67°38'W	17.Porto Acre AC	Terra firme forest	*C. guianensis*	4	F
10°01'S 67°41'W	18.Rio Branco AC	Varzea forest	*C. guianensis*	6	F
10°02'S 67°40'W	19.Rio Branco AC	Terra firme forest	*C. guianensis*	7	F

*: Numbers used in the distribution map (*Fig. 1b*).
†: Species identification based on presence of composite or simple leaves at seedling stage.

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Table 2. – Chloroplast DNA haplotypes in *Carapa guianensis* based on presence (1) or absence (0) of the least common variable band for the primer pairs and restriction enzymes used.

Haplotype	Primer pairs and restriction enzymes used						
	trnH-trnK* *Mse*I	trnK-trnK* *Hinf*I	trnK-trnK* *Mse*I	trnK-trnK* *Taq*1#	trnC-trnD * *Hinf*I	trnD-trnT * *Alu*I	trnF-trnVr † *Hinf*I
A	0	0	0	0 0	0	0	0
B	1	1	1	0 0	1	0	0
C	0	0	0	0 1	0	1	1
D	0	0	0	0 1	0	0	0
E	1	0	1	0 0	1	0	0
F	0	0	0	1 0	0	0	0

*: Primer sequence from DEMESURE *et al.* (1995).
†: Primer sequence from DUMOLIN-LAPEGUE *et al.* (1997).
#: The digestion of trnK-trnK with *Taq*1 gives two markers.

Table 3. – Global genetic differentiation and diversity levels at chloroplast DNA among populations of *Carapa guianensis* in the Amazon basin.

Type of measures	Parameters	Values (SD) for natural populations (7 locations)	Values (SD) for all populations (11 locations)
Unordered alleles	G_{st}	0.96 (0.04)	0.98 (0.02)
	H_S	0.03 (0.03)	0.02 (0.02)
	H_T	0.77 (0.06)	0.82 (0.02)
Ordered alleles	N_{st}	0.94 (0.07)	0.97 (0.03)
	V_S	0.04 (0.04)	0.02 (0.02)
	V_T	0.69 (0.13)	0.73 (0.11)
	D_m	3.0	3.8
	D_{wm}	2.7	3.4

chosen. Eight primer pairs gave no or only weak amplification products, and these were not used in subsequent analyses. Of the eight chloroplast PCR products tested, three were apparently monomorphic with the enzymes tested, and therefore were excluded in subsequent analyses, leaving a total of five polymorphic PCR products.

Data analysis

The Neighbour-Joining (NJ) tree of haplotypes was constructed using PAUP* (SWOFFORD, 2003), with the characters (restriction sites, indels) coded as present or absent, and distance expressed as mean character distance. Scoring haplotypes as ordered and unordered alleles respectively, we estimated levels of genetic differ-

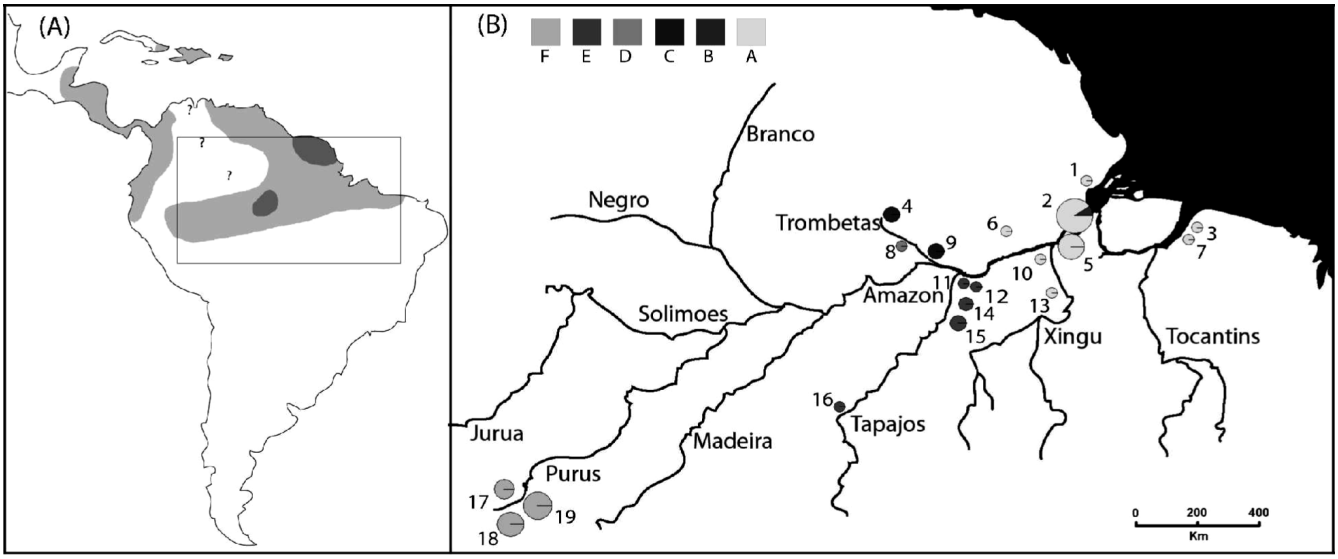

Figure 1. – (a) Geographic range of *Carapa guianensis* (light gray) and areas where both *C. guianensis* and *C. procera* are found (dark gray). (b) Map of the 19 locations sampled for chloroplast DNA variation in *Carapa guianensis* in the Amazon basin. Each location is represented by a numbered circle of size proportional to the number of individuals sampled, and each haplotype is represented by a different color (see *Table 1* for details).

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entiation (G_{st} and N_{st}), average within-location diversity (H_S and V_S), and total diversity (H_T and V_T) using the software PERMUT (PONS and PETIT, 1995; 1996). We also calculated the mean number of differences between haplotypes (D_m) and the weighted mean number of differences between haplotypes (D_{wm}). For these analyses, locations with a single individual assessed for cpDNA variation were omitted, however Belem and Santa Barbara, and the 2 Curua-Una locations, were treated as two synthetic locations because they were separated by less than 100 km. Since we can not rule out anthropogenic seed transfer in the plantation samples analyzed, we conducted two separate sets of analyses, one including all the 11 locations, and another including only the 7 locations of "natural" origin.

Results

The amplification of cpDNA regions from dried herbarium specimens failed, likely because of the low quality of the DNA extracted from these samples. Only short sequences < 500 bp could be amplified (results not shown).

A total of 53 samples from 19 sites yielded DNA amenable to PCR. Chloroplast variation is described in *Tables 1, 2* and *3*. Six polymorphisms were observed – four indels and two restriction site variants. The geographic distribution and phylogenetic relationship of these haplotypes is shown in *Figure 1* and *Figure 2*. A single haplotype was found in all locations except Gurupa-Island, which is an island on the Amazon River with floodplain varzea forest. The analysis of genetic differentiation is presented in *Table 3*.

Discussion

Contrary to our hypothesis that hydrochory has played a major role in determining phylogeographic patterns in this species, the general pattern of cpDNA variation (*Figure 1*) is consistent with limited seed dispersal at the scale of the Amazon basin. The global genetic differentiation measures (*Table 3*) are higher than the average reported in plant species (PETIT *et al.,* 2005), which suggests that gene flow is limited. This may be explained by the fact that hydrochory does not result in isotropic gene flow on a large scale, and that most individuals in upland sites do not have opportunity to exchange seed over long distances. There is, however, limited evidence that hydrochory allows some exchange between populations, since the only sampled location exhibiting within-site variation in cpDNA is a flooded *varzea* population situated on an island in the Amazon River ("Gurupa-Island") (*Table 1*). But while the observed mixing of haplotypes is evidence that hydrochory may influence the genetic diversity of *Carapa guianensis* populations, this influence appears to be localized and does not seem to have promoted extensive genetic mixing. Although we analyzed only a limited number of individuals per site (for 10 sites a single individual was analyzed) our results suggest that most locations are fixed for a single haplotype, as reported for other species (*e.g.,* CAVERS *et al.,* 2003). More locations will need to be analyzed to test whether downstream locations could be locations of genetic mixing in this hydrochorous tree species.

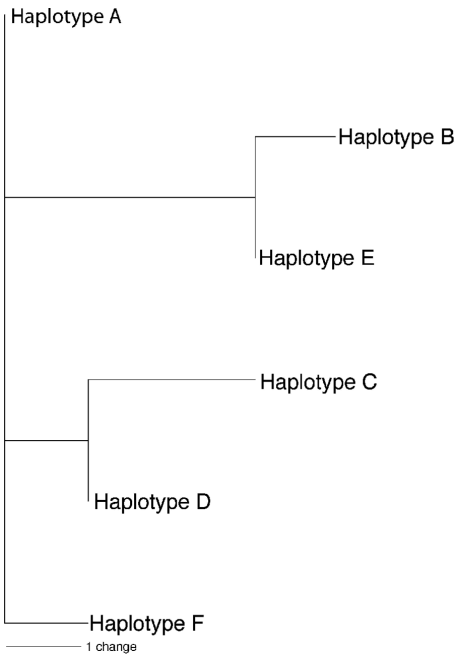

Figure 2. – Neighbor-joining tree of *Carapa guianensis* chloroplast haplotypes.

Several historical hypotheses have been put forward to explain patterns of biodiversity in the Amazon basin, and may help to explain the pattern of cpDNA variation found in this study. These include the presence of ecological gradients (ENDLER, 1982), the dynamics of Amazonian rivers (SALO *et al.,* 1986), past climatic changes with retreat of forest habitat to forest refuges (HAFFER, 1969), and the glacial cycles with sea level changes causing marine incursion into Amazonia (VUILLEUMIER, 1971; NORES, 1999). The last two hypotheses deserve some consideration here.

In Eastern Amazonia, there is evidence from studies of lake pollen that forest regression and savanna expansion occurred during the Pleistocene (ABSY *et al.,* 1991; VAN DER HAMMEN and ABSY, 1994), suggesting that reduction of rainfall in the past converted areas presently receiving less than 2000 mm of rain per year into savanna. If this climatic reconstruction is correct, recently recolonized zones could harbor a subset of cpDNA diversity that were present in the neighboring refugial areas. From our results, it can be seen that the total chloroplast diversity in the dry zones putatively "recolonized" does not appear lower ($H_T = 0.60$; $V_T = 0.60$; $D_{wm} = 6.0$) (8 locations) than the diversity in the combined putative refugial areas ($H_T = 0.62$; $V_T = 0.22$; $D_{wm} = 1.3$) (11 locations). However our sample size is small, and additional samples would be required to rigorously test this hypothesis. But our preliminary results do not support the hypothesis that a Pleistocene "refuge" Amazonian forest along the Atlantic coast has acted as the main source of recolonization for the interior by *Carapa guianensis*.

The phylogeny and geographic distribution of the haplotypes suggest that this species has colonized the Amazon basin from multiple sources (*Figures 1* and *2*). Large

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areas with low topography were flooded during past marine introgressions into Amazonia that occurred during the Tertiary and the Quaternary (NORES, 1999). Interestingly the distribution of haplotypes "C" and "E", differentiated by at least six independent mutations, corresponds roughly to high elevations areas of the Amazon basin, respectively the Guyanan shield in the North, and the Brazilian shield in the South. Therefore these areas may have been historical "refuges" for *Carapa guianensis* or may represent two independent colonization events. Conservation and management efforts should take into account the presence of such cryptic genetic differences in the Amazon basin in *Carapa guianensis*.

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