Biological Journal of the Linnean Society, 2009, **98**, 278–290. With 3 figures

Contrasting patterns of genetic structure in *Caryocar* (Caryocaraceae) congeners from flooded and upland Amazonian forests

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Received 24 February 2009; accepted for publication 23 April 2009

In the present study, we compare the genetic structure of a flooded forest tree *Caryocar microcarpum* and a *terra firme* forest tree *Caryocar villosum* in the lower Rio Negro region and test the hypothesis that the Rio Negro, the largest tributary on the left bank of the Amazon River, has been acting as a geographical barrier to gene flow between populations from the left and right banks. Seventeen adult individuals on the left bank and 27 on the right bank of Rio Negro were sampled for *C. microcarpum*, whereas 27 on the left and 20 on the right bank were sampled for *C. villosum*. Two chloroplast DNA regions were sequenced: the intron of *trnL* gene and the intergenic region between *psbA* and *trnH* genes; and all individuals were genotyped using ten microsatellite loci. The *trnL* intron and *psbA-trnH* intergenic spacer generated fragments of 459 bp and 424 bp, respectively. For *C. microcarpum*, six haplotypes were identified for *trnL* and seven for *psbA-trnH*. By contrast, only one haplotype was found for *C. villosum* for both sequences. The results obtained showed that the Rio Negro has not been a barrier to gene flow by pollen and seeds for either species. No genetic differentiation and a high migration rate between populations from the left and right banks of the Rio Negro were detected for the chloroplast sequences and nuclear microsatellites, for both *C. villosum* and *C. microcarpum*. Although the two analysed sequences showed a sharp topology difference, both indicated that multiple lineages may have contributed to the origin of *C. microcarpum* populations in the Rio Negro basin. Nevertheless, for *C. villosum*, from *terra firme*, the results obtained may provide evidence of a recent expansion of one maternal lineage from an ancient relic population surviving in one of the few moist forest refuges of the Guiana Shield during extended droughts of the glacial periods. We hypothesize that the contrasting environments colonized by this congener pair may have played an important role in shaping the genetic structure of both species.

ADDITIONAL KEYWORDS: Amazonia – *Caryocar microcarpum* – *Caryocar villosum* – Caryocaraceae – cpDNA – microsatellites – Neotropical tree – population genetic structure – riverine barrier hypothesis.

INTRODUCTION

The distribution and composition of vegetation in the Amazon have been deeply influenced by the drier episodes of the Tertiary and Pleistocene (Burnham & Graham, 1999; Vélez *et al.*, 2006), with large-scale replacement of the tropical rain forest by drought-tolerant dry forests and savannas mainly in southern, central, and eastern Amazonia (Hooghiemstra & Van der Hammen, 1998; Pennington *et al.*, 2000, 2004;

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Van der Hammen & Hooghiemstra, 2000; for an alternative view, see Colinvaux, Oliveira & Bush, 2000). The long series of ice ages may have led to extinctions but, concomitantly, may have stimulated the evolution and speciation of other groups (Van der Hammen, 1974; Hooghiemstra & van der Hammen, 2004). The altitudinal gradient may have allowed the invasion of montane plant species into the basin during the cooler periods and the evolution and adaptation of other groups (Hoorn *et al.*, 1995; Hooghiemstra & Van der Hammen, 1998, 2004; Colinvaux *et al.*, 2000; Urrego *et al.*, 2006). Proxy data also indicate a dynamic changing on the Amazon River and its tributaries, especially subsequent to the Miocene (Hooghiemstra & Van der Hammen, 1998). On the other hand, sea level elevations during the Tertiary, especially in the Miocene, caused the flooding of extensive Amazonian lowlands, isolating populations at higher elevations, mainly in the Guianan Shield, Brazilian Shield, and the base of the eastern slope of the Andes, changing the landscape and affecting species distribution (Webb, 1995).

Analyses of paleodunes show that the Rio Negro may have attained lower levels during the glaciation periods of the Tertiary (Carneiro-Filho *et al.*, 2002) and paleogeographic reconstruction has revealed Quaternary changes in the Rio Negro channel (Silva *et al.*, 2007). Hence, populations on both banks of the Amazon River itself and its tributaries may have been closer, or in contact, during some periods of the Quaternary. Nevertheless, the riverine barrier hypothesis, which had its own origin in Wallace's paper 'On the monkeys of the Amazon' (Wallace, 1852) has been raised to explain the megadiversity of Amazonia (Haffer, 1997). The main prediction of this hypothesis is that the Amazon River and its major tributaries have acted as geographical barriers, leading to vicariance speciation in many groups. Another prediction of this hypothesis is the occurrence of higher genetic differentiation among populations on opposite river banks. The relationship of dispersal and riverine barriers is not so clear cut (Colwell, 2000). For many groups, riverine barriers could not account for the observed patterns of species richness, geographical distribution, and differentiation (*Ateles*, Primata: Collins & Dubach, 2000; mammal species: Patton, Da Silva & Malcolm, 2000; avian species: Bates, Haffer & Grismer, 2004; passerine birds: Hayes & Sewlal, 2004), but could account for other groups (frogs and mammals: Gascon, Malcolm & Patton, 2000; mammals: Patton *et al.*, 2000; Riodinid butterflies: Hall & Harvey, 2002; passerine birds: Hayes & Sewlal, 2004).

Comparative analysis of nuclear and organelle genomes, with different modes of inheritance, mutation, and evolutionary rates, may provide a powerful analysis of the role of historical and contemporary events on current species distributions and genetic structure. For plants, the analysis of nuclear and chloroplast genomes may also clarify the relative importance of pollen and seed flows on population structure (McCauley, 1995; Schaal *et al.*, 1998; Collevatti, Grattapaglia & Hay, 2003). Moreover, analysis of different regions of the chloroplast genome, which display different mutation rates, may provide additional insights about the evolution and historical spread of populations (Soltis & Soltis, 1998). The genetic structure of the nuclear genome may be caused by historical and contemporary gene flow, by differential selection among habitats, genetic drift, and the mating systems that determine inbreeding effects on population differentiation (Wright, 1931). Nevertheless, genetic structure of the organelle genome, usually uniparentally inherited, is more affected by historical relationships and gene flow and by demographic fluctuations caused by historical events such as glaciations and climatic fluctuations over a geological time scale (Avise, 1994; Schaal *et al.*, 1998). Additionally, because of the haploid nature and mode of inheritance, the effective population size of the chloroplast genome is one-half the size of the nuclear genome, leading to a stronger effect of genetic drift on population genetic structure (Ennos, 1994).

Caryocar villosum Aublet (Caryocaraceae), popularly known as piquiá, is a low-density widely-distributed Amazonian emergent tree species, up to 50 m tall, in the upland (*terra firme*) forests, whereas *Caryocar microcarpum* Ducke, known as piquiarana (false piquiá in the Tupi language), is a 25-m tall, habitat-specific tree, growing in the seasonally-flooded blackwater forest (*igapó forest*), widespread throughout the Guianas and Northern Amazonia (Prance & Freitas da Silva, 1973). The main goal of the present study was to compare the genetic structure of this congener pair adapted for contrasting forest habitats in the lower Rio Negro region. Additionally, we tested the hypothesis that the Rio Negro is a relevant geographical barrier for pollen and seed flows for both species. The results are discussed in the light of the climatic and hydrographic changes that occurred during the Plio-Pleistocene period in the lower Rio Negro region. The genetic structure of *C. villosum* and *C. microcarpum* was studied based on the polymorphism at two chloroplast DNA regions and on ten nuclear microsatellite loci.

MATERIAL AND METHODS

POPULATIONS, SAMPLING, AND DNA EXTRACTION

The Rio Negro, 1700 km long, is the largest left bank tributary of the Amazon River and the largest black-

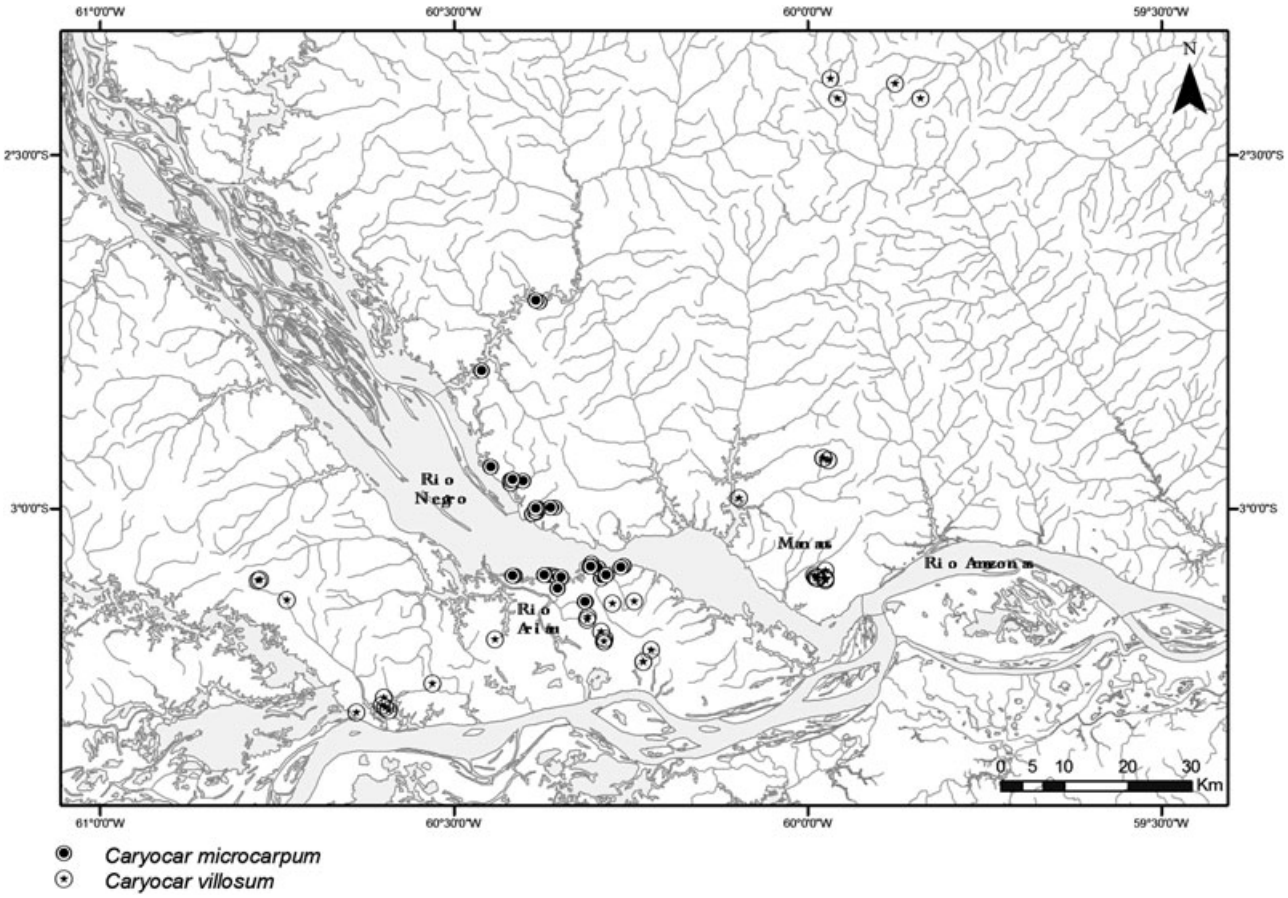

Figure 1. Sample sites of *Caryocar villosum* and *Caryocar microcarpum* individuals on the lower Rio Negro. The Iranduba region which was part of the left bank of the Rio Negro is the land mass delimited by the Rio Ariau (West) and the Rio Negro (East).

water river in the world. In its lower stretch, the river width varies between 2 km (near its mouth in Manaus) to 24 km in the Anavilhanas Archipelago region. The study site lies in the lower Rio Negro region, from its mouth (where the Rio Negro joins the Amazon River) to approximately 50 km upstream. The Rio Negro width varies in the range 2–12 km in this area (Fig. 1).

Twenty-seven adult individuals of *C. villosum* were sampled on the left bank and 20 on the right bank. For *C. microcarpum*, 17 adult individuals were sampled on the left bank and 27 on the right (Fig. 1). Distance between pairs of individuals varied between 300 m to approximately 75 km for *C. microcarpum*, and 150 m to approximately 150 km for *C. villosum* (Fig. 1). All individuals were mapped using a GPS (geographical positional system) and expanded leaves were collected and stored at –80 °C. Genomic DNA extraction followed the standard CTAB procedure (Doyle & Doyle, 1987).

Ten individuals of *Caryocar brasiliense* Camb., from a single population at Mato Grosso (Cerrado biome), 1225 km south-east of Manaus, were also sampled and included as an outgroup in the phylogeographical analysis based on chloroplast genome.

Chloroplast sequencing analysis

Two fragments corresponding to noncoding regions of chloroplast (cp)DNA were sequenced for the two studied species and for the outgroup: the intron of the *trn*L gene, using the 'c–d' pair of primers (Taberlet *et al.*, 1991), and the intergenic region between *psbA* and *trnH* genes (Azuma *et al.*, 2001). Fragments were amplified by the polymerase chain reaction (PCR) in a 20-mL volume containing 1.0 mM of each primer, 1 unit of Taq DNA polymerase (Phoneutria, BR), 250 mM of each dNTP, 1 × reaction buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$), 2.0 mg of bovine serum albumin (BSA) and 10.0 ng of template DNA. Amplifications were performed using a GeneAmp PCR System 9700 (Applied Biosystems) with the conditions: 96 °C for 2 min (one cycle); 94 °C for 1 min, 56 °C for 1 min, 72 °C for 1 min (30 cycles);

and 72 °C for 10 min (one cycle). PCR products were sequenced on an ABI Prism 377 automated DNA sequencer (Applied Biosystems) using DYEnamic ET terminator cycle sequencing kit (GE Healthcare), according to the manufacturer's instructions. Both fragments were sequenced in forward and reverse directions. Sequences were aligned using CLUSTALX (Thompson *et al.*, 1997), and characters (each base pair) were equally weighted before analysis. In addition, alignment was improved by hand *sensu* Kelchner (2000). For the intron of *trnL* gene, the secondary structure was determined for each haplotype to verify the effect of indels and substitutions on molecule stability using MFOLD (Zuker, 2003) and to guide hand alignment.

Intraspecific phylogenies for sequencing data were inferred using median-joining network analysis (Bandelt, Forster & Röhl, 1999) performed with the software NETWORK, version 4.2.0.1 (Forster, Bandelt & Röhl, 2004). In this analysis, a minimum spanning network was constructed, based on the union of all minimum spanning trees (Bandelt *et al.*, 1999). Using parsimony criteria, the software finds the median vectors (i.e. the consensus sequences of mutually close sequences, biologically interpreted as possible unsampled sequences or extinct ancestral sequences) (Bandelt *et al.*, 1999).

An analysis of molecular variance (AMOVA; Excoffier, Smouse & Quattro, 1992) was performed using ARLEQUIN, version 2.000 (Schneider, Roessli & Excoffier, 2000) to test the hypothesis of genetic differentiation among individuals from the left and right banks. The population genetic structure parameter θ (Weir & Cockerham, 1984) was estimated from AMOVA. Significance of θ was tested by a non-parametric permutation test (Excoffier *et al.*, 1992) implemented in the ARLEQUIN.

To better understand phylogenetic relationships, the hypothesis that the current pattern of haplotype diversity and distribution was caused by a bottleneck followed by a sudden expansion of the population was tested. The mismatch distribution was obtained using the DNASP, version 4.10.9 (Rozas *et al.*, 2003) and the hypothesis was tested using the Harpending's Raggeness Index R (Rogers & Harpending, 1992; Schneider & Excoffier, 1999) and Fu's F-test (Fu, 1997), also using DNASP.

A coalescent model (Kingman, 1982) was used to distinguish the contribution of isolation and migration with the observed patterns of genetic divergence between populations from the left and right banks, and to better understand the demographic history of the species. The demographic parameters $\theta = 2mN_e$ (coalescence force or mutation parameter), g [growth force, or exponential growth rate $g = \theta \exp(-gt)$, where t is time to coalescence is the mutational unit] and $M = 2N_e m/\theta$ (migration force or immigration rate) were estimated based on a maximum likelihood estimation using Markov chain Monte Carlo approach (Beerli & Felsenstein, 2001) implemented in LAMARC, version 2.0.2 (Kuhner, 2006). Four independent analyses were run with ten initial chains and two final chains for the combined data sequences.

Microsatellite analysis

Ten microsatellite loci, previously developed for *Caryocar brasiliense* and transferred to other Caryocaraceae species (Collevatti, Brondani & Grattapaglia, 1999), were used to genotype the sampled individuals. Forward primers were labelled with a fluorescent dye. Four loci (cb01, cb06, cb09, and cb12) were labelled with 6-FAM, three with HEX (cb03, cb11, and cb13), and three with NED (cb05, cb20, and cb23). PCR amplifications were performed in a 10-mL volume containing 0.5 mM of each primer, 1 unit of Taq DNA polymerase (Phoneutria), 200 mM of each dNTP, 1 × reaction buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$), 2.0 μg of BSA and 10.0 ng of template DNA. Amplifications were performed using a GeneAmp PCR System 9700 (Applied Biosystems) with the conditions: 96 °C for 2 min (one cycle); 94 °C for 1 min, 56 °C or 54 °C for 1 min (according to each primer), 72 °C for 1 min (30 cycles); and 72 °C for 1 h (one cycle). Reactions were then diluted 1 : 5 and electrophoresed in 5% denaturing polyacrylamide gel in an ABI Prism 377 automated DNA sequencer (Applied Biosystems). Fluorescent PCR products were automatically sized using Genescan and Genotyper softwares (Applied Biosystems).

Microsatellite loci were characterized for number of alleles per locus and observed and expected heterozygosities under Hardy–Weinberg equilibrium, and inbreeding coefficients (f), for each locus and over all loci (Nei, 1978). Analyses were performed with FSTAT, version 2.9.3.2 (Goudet, 2002) and randomization based tests with Bonferroni correction were performed generating the log-likelihood statistics G to test for deviation from Hardy–Weinberg expectations (Goudet *et al.*, 1996).

Genetic differentiation between populations of each bank was assessed by Wright's F-statistics, F, θ, and f (Wright, 1951), obtained from an analysis of variance of allele frequencies (Cockerham, 1969; Weir & Cockerham, 1984) and by Slatkin's R_{ST} (Slatkin, 1995) obtained from an analysis of variance of allele size *sensu* Goodman (1997). The analyses were performed using FSTAT, version 2.9.3.2 (Goudet, 2002). A significance test of differentiation with Bonferroni correction was performed by randomizing genotypes among samples to obtain the log-likelihood G statistics (Goudet *et al.*, 1996). Additionally, a Bayesian

analysis of population structure was carried out for each species with STRUCTURE, version 2.2 (Pritchard, Stephens & Donnelly, 2000) to verify whether sampled individuals comprise a single gene pool or if they belong to different demes or populations, without prior geographical information. With a burn-in period of 100 000 generations and 100 000 steps of Markov chain Monte Carlo simulations, lnPr(*X*/*K*), F_{ST} and *Q* (individual ancestry) were estimated for different values of *K* (number of populations) in the range 1–10. Admixture model and correlated allele frequencies were considered for all analyses. For each *K*-value, 20 runs were carried out to verify the consistency of the results.

The influence of recent bottleneck on the pattern of genetic variation was also analysed using BOTTLENECK software (Cornuet & Luikart, 1996). Populations that have experienced recent bottleneck will present observed gene diversity (or expected heterozygosity under Hardy–Weinberg equilibrium) higher than that expected under mutation–drift equilibrium, Heq (Maruyama & Fuerst, 1985; Luikart *et al.*, 1998). Distribution of Heq and the standard deviation were obtained, based on a simulation of a coalescent process of the ten loci, using 10 000 interactions, under the stepwise mutation model (Cornuet & Luikart, 1996) and under the mixed model (70% under stepwise mutation model and 30% under two phase model; Di Rienzo *et al.*, 1994). Mutation–drift equilibrium heterozygosity overall loci was compared with the observed one (H_E, heterozygosity under Hardy–Weinberg equilibrium) to verify whether there is heterozygosity excess by the Wilcoxon sign rank test. Additionally, allele frequency distribution was generated to verify bottleneck signatures on the shape of frequency distribution.

Demographic parameters were estimated for microsatellite data based on the coalescent model (Kingman, 1982) as described above for sequencing data, correcting for diploid genome ($\theta = 4mN_e$ and $M = 4N_em/\theta$).

RESULTS

Chloroplast sequences

Amplification of the noncoding *trnL* intron and *psbA-trnH* intergenic spacer generated fragments of 459bp and 424bp, respectively. All sequences were submitted to GenBank database (accession numbers EU339829 to EU339929, for *trnL*; and EU350258 to EU350358, for *psbA-trnH*).

Populations of *C. microcarpum* and *C. villosum* from the lower Rio Negro exhibited a contrasting pattern of polymorphism in the chloroplast genome. Although *C. microcarpum* showed a high within population polymorphism, with several haplotypes co-occurring on both banks, *C. villosum* presented no polymorphism at all for the two chloroplast regions analysed, even though some individuals are more than 150 km apart.

For the *trnL* intron, one substitution site (character CH113, C/A) and two indels were found (CH111 and CH328), and an indel or a substitution (T/C) could be found on CH112. Additionally, a 26-bp deletion (CH140 to CH165) was found for three individuals of *C. microcarpum* from the right bank. This deletion was considered as a unique mutation event for all statistical analyses. The *trnL* fragment is characterized by a high abundance of adenine (31%) and thymine (40%), and the presence of many mononucleotide repeats along the sequence.

The intergenic region between *psbA* and *trnH* genes was also characterized by a high abundance of adenine (33%) and thymine (42%). Thirteen substitution sites were found, as well as many repetitive regions. The longest was composed by $(T)_8C(T)_{19}A(T)_3$. Polymorphisms at this repeated region were not considered for phylogeographical analysis because of uncertain homology relationships derived from ambiguous alignment. Long insertions/deletions were also found in *C. microcarpum*. A long deletion (96 bp) was found in 18 individuals (CH190 to CH286) and a 7-bp deletion was found in 57 individuals (CH174 to CH180). All long insertions/deletions were considered as unique mutation events for all statistical analyses.

For the *trnL* fragment, only one haplotype was found for the 47 individuals of *C. villosum* from right and left banks of Rio Negro (Table 1), and six different haplotypes could be distinguished in 44 individuals of *C. microcarpum* (Table 1). Six individuals of *C. brasiliense*, out of ten, showed the same haplotype of *C. villosum* (Table 1). Six mutations explained the median-joining network based on the *trnL* intron sequence (Fig. 2). Haplotype MMR40R was the most frequent for *C. microcarpum* ($N = 28$), and was presented in individuals from the right and left banks (Fig. 2 and Table 1).

When the *psbA-trnH* fragment was analysed, again, only one haplotype was found for *C. villosum* (Table 1), but seven different haplotypes could be distinguished for *C. microcarpum* (Table 1). Six individuals of *C. brasiliense* presented one haplotype in common with *C. villosum* and *C. microcarpum* (Table 1). Twenty-one mutations explained the median-joining network based on the *psbA-trnH* sequence (Fig. 3). Individuals from the left and right banks of the Rio Negro presented the same haplotypes for both species (Fig. 3).

Some differences in *trnL* and *psbA-trnH* network topologies could be found. Although the number of haplotypes for *C. microcarpum* was very similar (six

Table 1. Haplotype frequencies on the left and right banks of the lower Rio Negro, based on 47 individuals of *Caryocar microcarpum* and 44 of *Caryocar villosum*, for the *trnL* intron and *psbA-trnH* intergenic regions of chloroplast DNA, with *Caryocar brasiliense* as outgroup

Region	Haplotype	*Caryocar microcarpum*		*Caryocar villosum*		*Caryocar brasiliense*	Total
		Left	Right	Left	Right		
trnL	VRD13L	0	0	27	20	6	53
	MSB28R	0	3	0	0	0	3
	MRA22R	0	2	0	0	0	2
	MPF02L	2	0	0	0	0	2
	MJA17L	4	0	0	0	0	4
	MMR38R	0	5	0	0	0	5
	MMR40R	11	17	0	0	0	28
	BTOF1	0	0	0	0	4	4
	Total	17	27	27	20	10	101
psbA-trnH	VRD14L	2	1	27	20	6	56
	MSB30R	0	4	0	0	0	4
	MRA22R	0	1	0	0	0	1
	MSO57R	0	2	0	0	0	2
	MAR13L	9	5	0	0	0	14
	MAR06L	4	4	0	0	0	8
	MAR14L	2	10	0	0	0	12
	BVTO	0	0	0	0	4	4
	Total	17	27	27	20	10	101

for *trnL* and seven for *psbA-trnH*) and some patterns of haplotype sharing were maintained, some differences were identified, mainly because of the split of the haplotype MMR40R (*trnL*) in several haplotypes in *psbA-trnH* (MSO57R, MAR13L, MAR06L, and MAR14L; Figs 2, 3 and Table 1).

No differentiation between populations of *C. microcarpum* from the right and left banks of the Rio Negro could be detected when the analysis of molecular variance was performed, for both *trnL* and *psbA-trnH* regions ($\theta = 0.0108$, $P = 0.24545$; $\theta = 0.0657$, $P = 0.752$, respectively). Additionally, there is no evidence of a sudden expansion subsequent to a bottleneck for the *C. microcarpum* population when we analysed mismatch distribution for *trnL* intron and *psbA-trnH* sequences (Table 2). The Fu neutrality test was significant only for *trnL* (Table 2). Coalescent analysis showed a high migration rate between populations from the left and right banks for *C. microcarpum* (Table 3). Additionally, the population from the left bank is expanding, whereas the population from the right bank is shrinking (Table 3).

Microsatellite analysis

All pairs of microsatellite loci were in linkage equilibrium for both species ($P > 0.001389$, for *C. microcarpum*; $P > 0.001111$, for *C. villosum*; *P*-values are Bonferroni adjusted for 5% nominal level). They all displayed high levels of polymorphism for both species (Table 4). Primer cb1 did not amplify for *C. microcarpum*. Pattern of observed and expected heterozygosity varied between species: for *C. microcarpum*, cb23 presented the highest genetic diversity and, for *C. villosum*, cb11 presented the highest genetic diversity (Table 4). Six loci presented observed heterozygosity that was significantly lower than expected under Hardy–Weinberg equilibrium for *C. microcarpum* but, for *C. villosum*, observed heterozygosity differed from that expected only for cb12 (Table 4).

No significant differentiation between populations from the left and right banks was found for either species ($\theta = 0.030$, $P = 0.004$, adjusted nominal 5% level with Bonferroni correction of 0.00278 for *C. microcarpum*, and $\theta = 0.006$, $P = 0.0385$, adjusted nominal 5% level with Bonferroni correction of 0.00250, for *C. villosum*). R_{ST} also showed a low level of genetic differentiation for both species ($R_{ST} = 0.0511$, for *C. microcarpum*, $R_{ST} = 0.0002$ for *C. villosum*). A significant amount of inbreeding was found for *C. microcarpum* ($f = 0.119$, $P = 0.0001$, adjusted nominal 5% level with Bonferroni correction of 0.00556), but not for *C. villosum* ($f = -0.002$, $P = 0.5650$, adjusted nominal 5% level with Bonferroni correction of 0.005). Bayesian analysis showed a weak population structur-

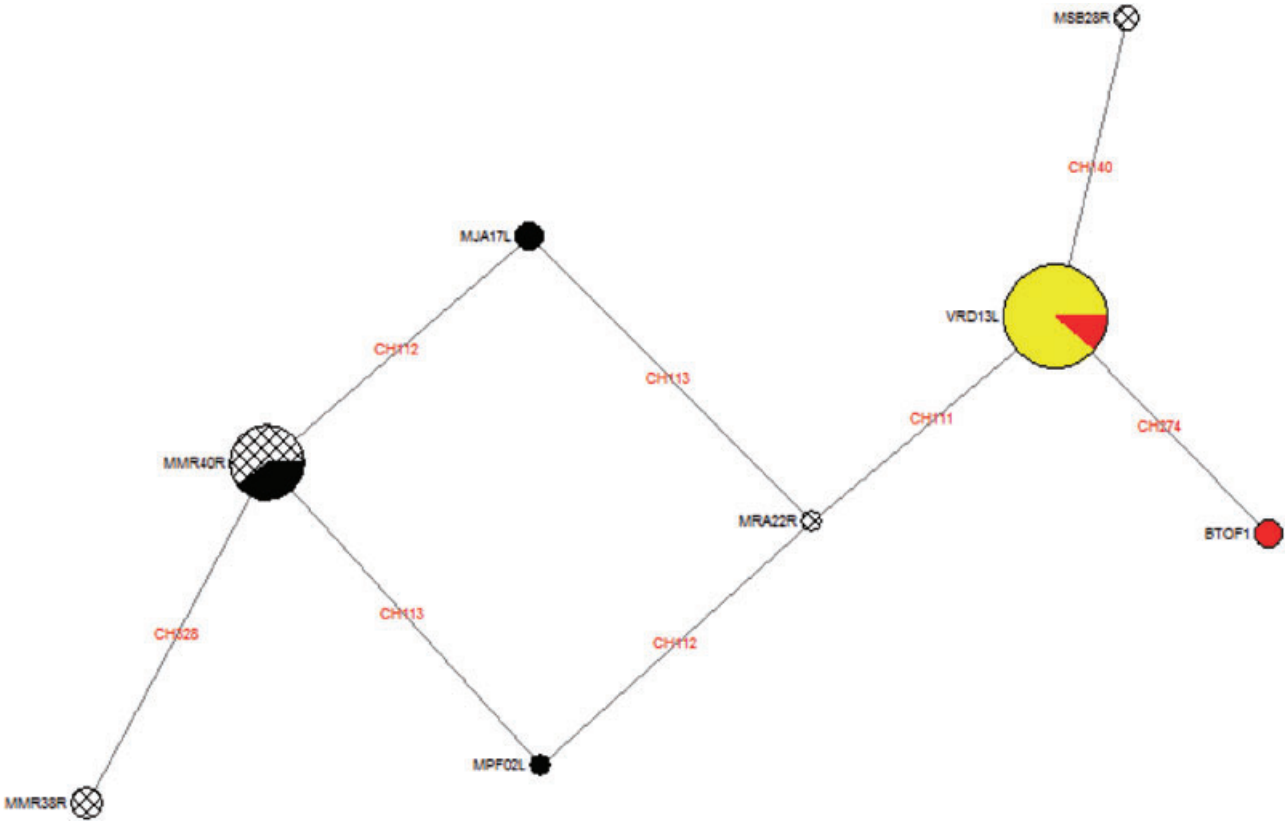

Figure 2. Median-joining network based on the sequence of the *trnL* intron for 47 individuals of *Caryocar microcarpum* and 44 individuals of *Caryocar villosum* from the lower Rio Negro. Circumference size is proportional to the haplotype frequency. Black, *C. microcarpum* left bank; white with diagonal black lines, *C. microcarpum* right bank; yellow (pale grey), *C. villosum* left and right banks; red (dark grey), outgroup species *C. brasiliense*. All mutations are shown, $\varepsilon = 0$. Mutation site CH140 represents a 26-bp deletion.

ing for both species for $K = 2$ [$\ln P(X/K) = -1194.3$ for *C. microcarpum* and $\ln P(X/K) = -1326.4$ for *C. villosum*], independent of the prior information on geographic sampling location. Nevertheless, mean values of F_{ST} were very low for all K-values, and the differences in $\Pr(K)$ were very small for both species (results not shown). In addition, the assignments were approximately symmetric to all populations (~1/K) and no individuals were strongly assigned, indicating that there is no population structure.

No evidence of recent bottlenecks were found for either species (Wilcoxon test, $P = 0.21289$ for *C. microcarpum*, $P = 0.1875$ for *C. villosum*). Additionally, both species presented the L-shaped allele frequency distribution, as expected by mutation–drift equilibrium, with most of the alleles with low frequency and a low number of alleles with high frequency. For *C. microcarpum* microsatellites, coalescent analysis showed also a higher migration from the left to the right bank compared to the opposite direction, although it was not as pronounced as that observed to the chloroplast sequences (Table 3). A high left-to-right migration was also obtained in the microsatellite coalescent analysis for *C. villosum* (Table 3). Additionally, for both *C. microcarpum* and *C. villosum*, microsatellite data showed that populations from the left bank are stationary and those from the right bank are shrinking (Table 3).

DISCUSSION

CONTRASTING POPULATION GENETIC STRUCTURES

The climatic changes during the late Pleistocene–Holocene transition influenced the distribution of species and dynamics of terrestrial and flooded habitats in the Amazon and other tropical regions of the globe (Anhuf *et al.*, 2006). The sequence of several glaciations led to an advance of savanna-like vegetation and dry forest and retreat of rainforest tree species, and the opposite in interglacial periods (Pennington, Prado & Pendry, 2000). These changes in the landscape may have caused forest fragmentation and isolation, leading to a bottleneck and loss of genetic

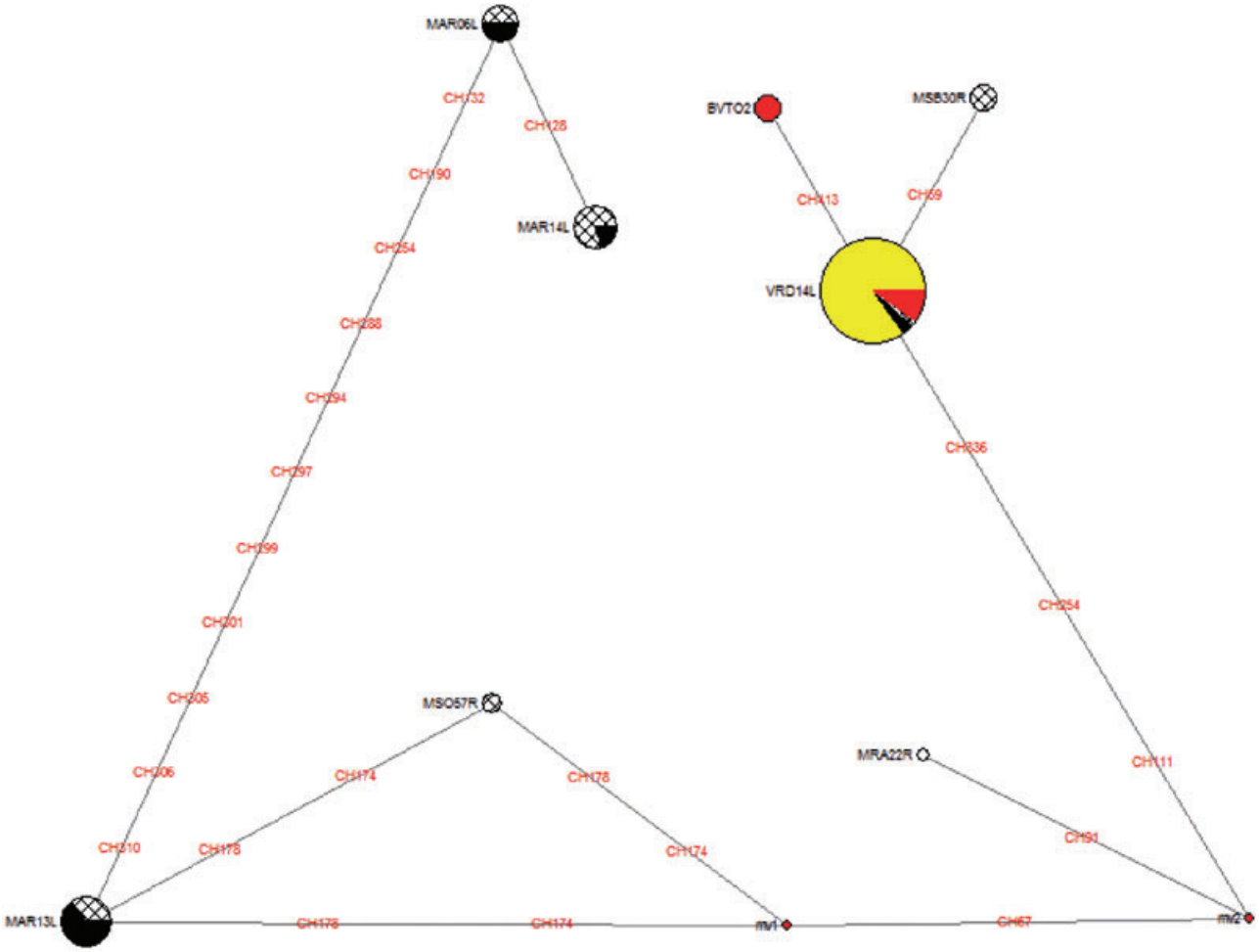

Figure 3. Median-joining network based on the sequence of the *psbA-trnH* intergenic region for 47 individuals of *Caryocar microcarpum* and 44 individuals of *Caryocar villosum* from the lower Rio Negro. Circumference size is proportional to the haplotype frequency. Black, *C. microcarpum* left bank; white with diagonal black lines, *C. microcarpum* right bank; yellow (pale grey), *C. villosum* left and right banks; red (dark grey), outgroup species *C. brasiliense*. All mutations are shown, ε = 0 (mv1 and mv2, median vectors). Mutation site CH91 represents a 5-bp duplication; CH190 represents a a 96-bp deletion; and CH174 represents a a substitution and a 7-bp deletion.

Table 2. Polymorphism and sudden expansion tests for *Caryocar microcarpum* populations from the right and left banks of Rio Negro, based on the polymorphism at *trnL* intron and *psbA-trnH* intergenic region

	trnL			*psbA-trnH*		
	Right	Left	Both banks	Right	Left	Both banks
H	1.0000 (0.0120)	1.0000 (0.0388)	1.0000 (0.0068)	0.8238 (0.0481)	0.7778 (0.0907)	0.8108 (0.0328)
π	0.0009 (0.0009)	0.0004 (0.0006)	0.0007 (0.0008)	0.1529 (0.0767)	0.1536 (0.0821)	0.1483 (0.0732)
HR	0.121	0.203	0.107	0.157	0.210	0.152
F	–3028.0*	–3482.2*	–3402.2*	4.761	3.667	6.292

Values in parentheses indicate the SD. Values of *F* and *HR* marked with an asterisk are significant: *$P < 0.005$.
H, haplotype diversity; π, nucleotide diversity; *HR*, Harpending's raggedness index; *F*, Fu' test of neutrality.

Table 3. Demographic parameters based on maximum likelihood estimation performed with LAMARC software for *Caryocar microcarpum* populations from the right and left banks of Rio Negro, based on the polymorphism at *trnL* intron and *psbA-trnH* intergenic region, and based on ten microsatellite loci for *C. microcarpum* and *Caryocar villosum* from each bank

	Caryocar microcarpum				*Caryocar villosum*	
	trnL and *psbA-trnH*		Microsatellites		Microsatellites	
Pop	Right	Left	Right	Left	Right	Left
θ	0.007	0.002	0.041	0.073	0.108	0.161
g	–51.449	6893.291	–228.360	–0.045	–127.335	0.171
m	25.103	1.270	1.218	0.458	1.454	0.236

θ, coalescent parameter; *g*, exponential growth parameter; *m*, number of migrants per generation ($2N_m$ for chloroplast and $4N_m$ for microsatellites).

Table 4. Characterization of the ten microsatellite loci based on 47 individuals of *Caryocar microcarpum* and 44 individuals of *Caryocar villosum* from the lower Rio Negro

	Caryocar microcarpum				*Caryocar villosum*			
Locus	*A*	H_O	H_E	*f*	*A*	H_O	H_E	*f*
cb1	–	–	–	–	11	0.894	0.902	0.009
cb3	18	0.697	0.910	0.190*	13	0.972	0.903	–0.074
cb5	18	0.879	0.903	0.103*	12	0.639	0.746	0.089
cb6	17	0.909	0.856	0.034	11	0.872	0.833	–0.094
cb9	11	0.909	0.864	–0.086	11	0.894	0.839	–0.074
cb11	21	0.727	0.815	0.036	18	1.000	0.933	–0.070
cb12	18	0.667	0.770	0.152*	16	0.761	0.927	0.192*
cb13	15	0.833	0.921	0.138*	12	0.794	0.861	0.057
cb20	15	0.439	0.557	0.352*	14	0.917	0.898	0.010
cb23	17	0.833	0.935	0.148*	12	0.922	0.867	–0.065
Overall loci	16.6	0.766	0.837	0.119*	13	0.867	0.871	–0.002

*Significant for $P < 0.00556$ (*C. microcarpum*) and $P < 0.005$ (*C. villosum*).
A, total number of alleles; H_O, observed heterozygosity; H_E, expected heterozygosity; *f*, inbreeding coefficient.

variability in tropical rainforest trees. After 7000 years BP, the climate became moister and populations restricted to refuges in the last glaciation may have spread and colonized favourable areas, attaining the present geographical distribution.

The remarkable differences in cpDNA diversity of this parapatric congeneric pair with distinct ecosystem preferences is an intriguing issue. Which historical and/or ecological factors have led to the evolutionary divergence of many intraspecific lineages in *C. microcarpum*, and only one in *C. villosum*, in the lower Rio Negro region? Because differences in evolutionary rates between congeneric species are unlikely, we hypothesize that this contrasting pattern of genetic diversity may reflect the differences in the habitat stability and population persistence experienced by each species during the late Pleistocene, which is a period described as being of high aridity in the lower Rio Negro (Carneiro-Filho *et al.*, 2002).

The sole matrilineal lineage of *C. villosum* in the lower Rio Negro region might reflect isolation of restrict populations in few *terra firme* refugia during glacial period followed by extinction of low-frequency haplotypes, and subsequent expansion, leading to the present-day distribution. On the other hand, populations of species from seasonally-flooded *igapo* forest, restricted to riverbanks, were likely not too severely affected because of the persistence of the main water courses and riverine habitats even during the drier Pleistocenic periods (Prance, 1973). Under this scenario, a more stable and diverse population of *C. microcarpum* than *C. villosum* would be expected. If the *terra firme* and *igapo* habitats had a distinct historical effect in the diversifi-

cation of tree populations, similar genetic patterns may emerge as additional congeneric species with contrasting habitat preferences are analysed in the region.

Although results on bottleneck analyses for microsatellites did not statistically support a recent bottleneck for *C. villosum*, comparative analyses show that this species exhibited lower polymorphism for microsatellite markers than *C. microcarpum*, and no polymorphism for cpDNA markers. Because the population effective size for the chloroplast genome is half the size of the nuclear genome, a more severe effect of demographic events on the chloroplast genome may be expected. Furthermore, the tests used to detect bottlenecks are based on the assumption that a bottleneck is a recent event and that the population is sampled just after the bottleneck when the population is expanding (Rogers & Harpending, 1992; Fu, 1997). Therefore, the observed pattern may be the outcome of a bottleneck because the population was not expanding (left bank) or was shrinking (right bank).

The sharing of haplotypes between *C. microcarpum*, *C. villosum*, and *C. brasiliense* (Figs 2, 3) may be the outcome of incomplete lineage sorting of polymorphic ancestral gene pool (recent speciation in the group) or introgression as a result of hybridization. Further in depth phylogenetic analysis is necessary to better understand the evolution of this group and clarify this result.

Rio negro as a geographic barrier

Despite the remarkable between-species differences in terms of cpDNA genetic polymorphism, no significant genetic differentiation was detected for the chloroplast genomes between populations from the left and right banks of *C. villosum* and between those of *C. microcarpum*. No differences between river banks were also found for each species using nuclear microsatellites, and coalescent analysis revealed high migration between the left and right banks for both species. Furthermore, Bayesian analysis showed, for both species, that individuals sampled on both banks most likely belong to one gene pool. Hence, the results obtained in the present study suggest that the Rio Negro does not appear to be an important barrier to gene flow by pollen and seeds for either species. For both species, the results of the present study show that individuals from each bank belong to subpopulations of a larger population. In addition, coalescent analysis provides evidence of biased gene flow from subpopulations from the left to the right bank, as a source-sink system, for both species.

The lack of genetic differentiation among trees on opposite river banks may be related to ecological factors such as the long-distance pollination and seed dispersal of *Caryocar* species. The nocturnal, brush-like flowers of *Caryocar* are pollinated primarily by glossophagine and *Phyllostomus* bats (Gribel & Hay, 1993; Martins & Gribel, 2007), with the latter comprising pollinators that could potentially cross large rivers. The large and hard drupes of *Caryocar brasilense* are ingested and probably dispersed through endozoochory by tapirs (*Tapirus terrestris*, Tapiridae) in Central Brazil (R. Gribel, unpubl. observ.) and the same likely occurs with the Amazonian species of *Caryocar*. The tapir is a large, mobile mammal with a high level of swimming ability that promotes long-distance seed dispersal in many drupaceous plants of the Neotropical forests (Fragoso, Silvius & Correa, 2003). Many drupes in the seasonally-flooded forest are registered as dispersed by fishes (Gottsberger, 1978) and ichthyochory may also be important to connect populations of *C. microcarpum* from opposite river banks.

The lack of differentiation between populations from opposite banks may be a result of the ancient geological morphostructure of the lower Rio Negro because a landmass that is currently on the right margin of the Rio Negro, where many individuals of both species were sampled, was in the left margin until the Quaternary. Paleogeographic reconstruction of the lower Negro River revealed that the ancient channel flowed along the Ariau depression toward the Rio Solimões, and that the Iranduba region was part of the Rio Negro left bank (Silva *et al.*, 2007). Quaternary sedimentation subsequent to the filling of the Ariau channel resulted in the diversion of the lower stretch of the Negro River to the east, flowing through the fault occupied currently by the Rio Negro mouth (Silva *et al.*, 2007; Fig. 1).

Whatever the reason (ecologic or geomorphologic), the congruence between the results from the cpDNA sequences and microsatellite data strongly support the hypothesis that the Negro River has not been a geographical barrier to gene flow by pollen and by seeds for either species. A lack of population differentiation was also found for other Amazonian species over wide areas, suggesting long-distance gene flow (e.g. *Pterocarpus officinalis*: Rivera-Ocasio, Aide & McMillan, 2002; *Symphonia globulifera*: Dick, Abdul-Salim & Bermingham, 2003; *Ceiba pentandra*: Dick *et al.*, 2007). By contrast, the Lecythidaceae *Coythophora alta* presented high population differentiation, with an F_{ST} almost equal to 1.0, over distances of a few kilometers (Hamilton, 1999).

The present study shows that historical and ecological factors leading to diversification can be explored by genetic studies on closely-related taxa with contrasting habitats preferences. In conclusion, we show that the congeneric comparative analysis of polymorphism in different regions of the chloroplast

genome and with nuclear microsatellites may provide novel insights into the role of ancient events on the current distributions of genetic variation of tropical tree populations, for which information is typically scarce.

ACKNOWLEDGEMENTS

We acknowledge the Universidade Católica de Brasília (UCB) and Instituto Nacional de Pesquisas da Amazonia (INPA) for financial support. We are grateful to Luiz de Souza Coelho and José Lima dos Santos for their help with field work and two anonymous reviewers for their helpful comments on the manuscript.

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