ORIGINAL ARTICLE

Phylogeography of the endangered rosewood *Dalbergia nigra* (Fabaceae): insights into the evolutionary history and conservation of the Brazilian Atlantic Forest

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The Brazilian rosewood (*Dalbergia nigra*) is an endangered tree endemic to the central Brazilian Atlantic Forest, one of the world's most threatened biomes. The population diversity, phylogeographic structure and demographic history of this species were investigated using the variation in the chloroplast DNA (cpDNA) sequences of 185 individuals from 19 populations along the geographical range of the species. Fifteen haplotypes were detected in the analysis of 1297 bp from two non-coding sequences, *trn*V-*trn*M and *trn*L. We identified a strong genetic structure ($F_{ST} = 0.62$, $P < 0.0001$), with a latitudinal separation into three phylogeographic groups. The two northernmost groups showed evidence of having maintained historically larger populations than the southernmost group. Estimates of divergence times between these groups pointed to vicariance events in the Middle Pleistocene (ca. 350 000–780 000 years ago). The recurrence of past climatic changes in the central part of the Atlantic forest, with cycles of forest expansion and contraction, may have led to repeated vicariance events, resulting in the genetic differentiation of these groups. Based on comparisons among the populations of large reserves and small, disturbed fragments of the same phylogeographic group, we also found evidence of recent anthropogenic effects on genetic diversity. The results were also analysed with the aim of contributing to the conservation of *D. nigra*. We suggest that the three phylogeographic groups could be considered as three distinct management units. Based on the genetic diversity and uniqueness of the populations, we also indicate priority areas for conservation.
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Introduction

The Brazilian Atlantic Forest is considered the second largest tropical forest in South America, covering an area of more than one million square kilometres along the Brazilian coast and extending to eastern Paraguay and north-eastern Argentina (Oliveira-Filho and Fontes, 2000). This biome includes both evergreen forest, which runs along the coastline covering mountain chains at low and medium elevations ($\leqslant 1000$ m), and semi-deciduous forest, extending across a plateau (usually >600 m) in the centre and south-eastern interior of Brazil (Morellato and Haddad, 2000; Oliveira-Filho and Fontes, 2000). It is one of the most diverse biomes in the world, with high species richness and endemisms, and is considered one of the most threatened ecosystems on Earth due to intense disturbance, having been reduced to only 7.5% of its original area (Myers *et al.*, 2000).

Despite an increase in research efforts in the past few years, the knowledge about the evolutionary history of the Atlantic Forest is still limited. Phylogeographical studies have shown that the Atlantic Forest has a complex history that resulted in large lineage divergences. North–south genetic discontinuities in the central part of the Atlantic Forest (from the Bahia to São Paulo states) were found in some animal species, such as small mammals (*Metachirus nudicaudatus*; Costa, 2003), lizards (*Gymnodactilus darwin*, Pellegrino *et al.*, 2005), sloths (*Bradypus variegates*; Moraes-Barros *et al.*, 2006), birds (*Xiphorhynchus fuscus*; Cabanne *et al.*, 2007) and canids (*Cerdocyon thous*; Tchaicka *et al.*, 2007). The genetic split found in these studies is located in a region between the Jequitinhonha and Doce Rivers (see Figure 1 for river locations). The separation of these lineages has been dated in some studies to the Pleistocene (Cabanne *et al.*, 2007; Tchaicka *et al.*, 2007) and even before the Pleistocene (Costa, 2003; Pellegrino *et al.*, 2005). The few phylogeographic studies in plants have also shown genetic discontinuities in the central part of the Atlantic Forest, but the location of these splits are more southern (southern Espirito Santo/northern Rio de Janeiro states, $\sim 21°$S), as in the legume trees (*Caesalpinia echinata*, Lira *et al.*, 2003; *Hymenaea courbaril*, Ramos *et al.*, 2009).

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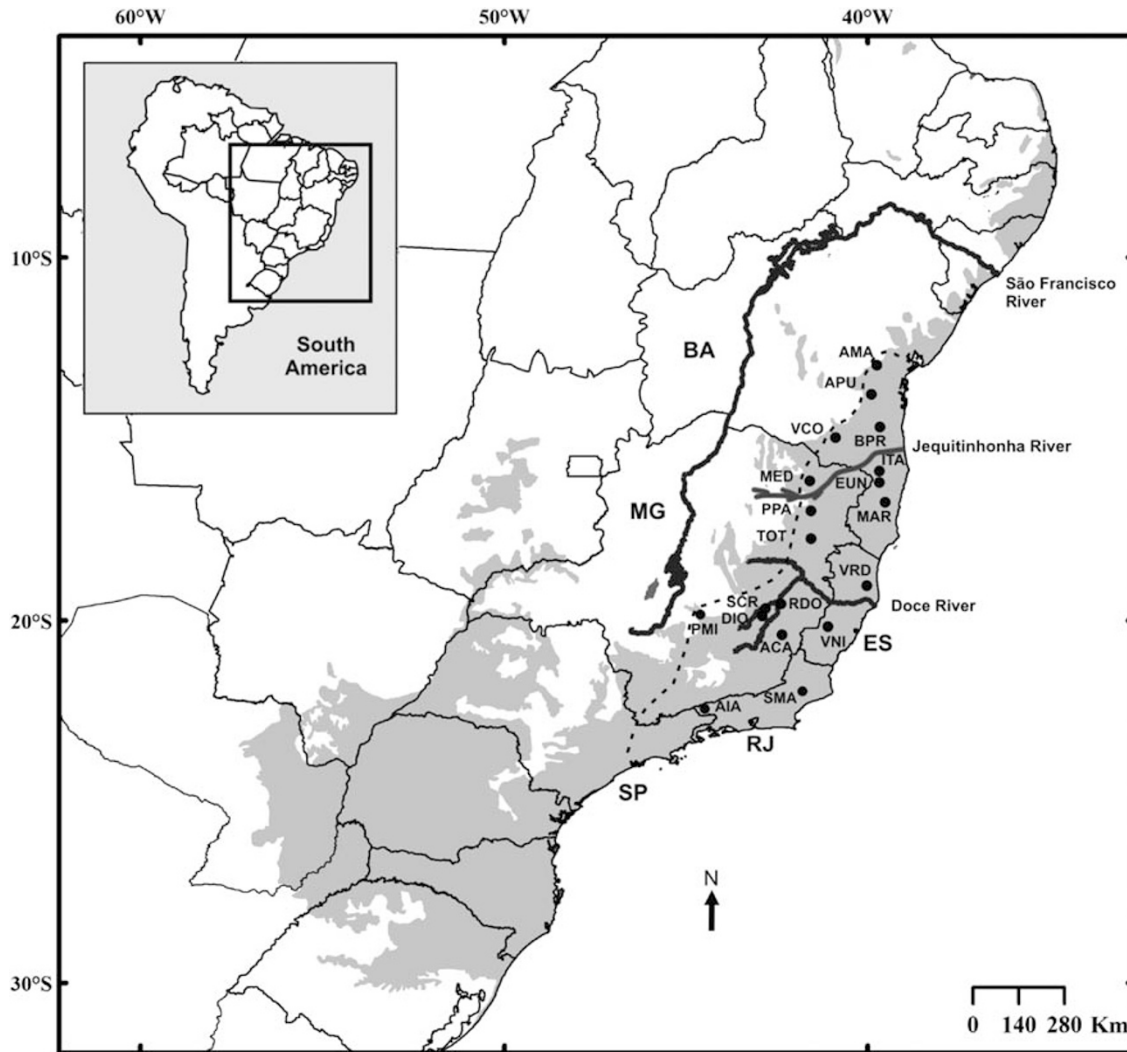E-mail: *lovatomb@icb.ufmg.br*

Figure 1 Map showing the geographical distribution of the 19 sampled populations for the phylogeographic study of *Dalbergia nigra* (black points) and the original distribution of the Brazilian Atlantic Forest in South America (grey). The approximate distribution limits of *D. nigra* are indicated by broken lines covering five Brazilian States: Bahia (BA), Minas Gerais (MG), Espírito Santo (ES), Rio de Janeiro (RJ) and São Paulo (SP). See Table 1 for the population abbreviations and other details.

However, in conifer trees (*Podocarpus sellowi, P. brasiliensis* and *P. lambertii,* Ledru *et al*., 2007) the split was observed at more northern locations (15°S). Although the studies in plants have attributed these genetic splits to climatic changes of the Quaternary, none has estimated the time since splitting. In addition, these phylogeographic studies with plants had a sparse sampling through the central Atlantic forest. Additional phylogeographic studies would help to reconstruct the evolutionary history of the central Atlantic Forest, improving conservation and management measures such as the identification of priority populations/areas for reserve implementation.

Vegetation changes in the Atlantic Forest related to climatic changes during the Pleistocene have been registered in paleopalinological studies, with the replacement of large areas of forests by subtropical grasslands and savannas during cooler and drier conditions (Behling 1995, 2002; Behling and Negrelle, 2001). Based on paleoclimatic models, Carnaval and Moritz (2008) predicted the presence of historically stable areas (refugia) in the Atlantic Forest during the Late Quaternary,

one large area in the central region (the Bahia refugium) and another smaller area (the Pernambuco refugium). These match the current centres of the endemism of several taxa and published phylogeographic studies based on animal mitochondrial DNA (mtDNA).

In addition to past historical events, it is known that human-mediated fragmentation and deforestation can also promote alterations in the diversity and genetic structure of species, frequently depleting genetic diversity within populations and increasing genetic differentiation among populations (Young *et al*., 1996; Aguilar *et al*., 2008). The high deforestation rate of the Atlantic Forest could lead to a loss of the signature of historical processes behind the genetic structure of species (Carnaval *et al*., 2009). Thus, studies that take into account both of the factors that affect the current genetic structure of species—historical and human-mediated events—are required to better understand the evolutionary process and establish effective conservation measures.

Here, we analyse the phylogeography of *Dalbergia nigra* (Vell.) Allemao ex Benth (Papilionoideae), known

as the Brazilian rosewood or jacarandá-da-Bahia, one of the most valuable tree species endemic to the central Atlantic Forest, which occurs from Bahia to the northern São Paulo States (Carvalho, 1994). This species produces a high-quality wood that is highly valued for the manufacture of musical instruments and fine furniture, thus resulting in its overcutting since the colonization of Brazil (1500 AD) (Carvalho, 1994). *D. nigra* is a threatened tree that is in the vulnerable category due to its over-exploration, the absence of replacement plantations and the deforestation of the Atlantic Forest (IUCN, 2008). *D. nigra* is extremely rare in nature, and its international trade has been prohibited since the 1990s by the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES, 2008).

We performed a dense sampling throughout the distribution range of *D. nigra* to obtain a more geographically detailed understanding of the evolutionary history of this endemic species of the central Atlantic Forest. Using chloroplast DNA (cpDNA) sequences, the phylogeographic structure and demographic history were investigated. In addition to classical phylogeographic methodologies, we performed a demographical analysis using the 'isolation with migration' model (IM, Hey and Nielsen, 2004, 2007), which is based on coalescent theory. This methodology provides estimates of the effective population sizes, time since the populations split and rate of migration after a population split even using a single locus (see Qiu *et al.*, 2009). This study was conducted to answer the following two questions: (1) What is the influence of the historical events of the Pleistocene on the current genetic diversity and structure of *D. nigra*, and specifically, are there genetic disjunctions along its distribution? (2) In addition to historical events, were the genetic diversities of populations affected by human-mediated fragmentation? The existence of a well-defined phylogeographic structure could be informative for understanding historical climatic events. A lower diversity in small forest fragments in relation to great reserve areas, both located in the same phylogeographic group, could be associated with more recent events, such as human-mediated fragmentation. Although cpDNA is not as powerful for detecting alterations in genetic structure in fragmented populations as more polymorphic markers, it can be expected that pronounced fragmentation results in a decrease in the genetic diversity of small populations, which could also be detected using cpDNA. All of the results obtained here were used to suggest conservation strategies for this endemic and threatened species of the Atlantic Forest.

Materials and methods

Study species and population sampling
Dalbergia nigra trees reach 15–25 m in height and 40–80 cm in diameter at breast height (Carvalho, 1994). It has a short flowering period (November–December) and a long fruiting period (January–September), both in supra-annual and irregular phenological cycles (data from southeast Brazil; Engel and Martins, 2005). *D. nigra* is pollinated by bees (personal observation), and its seeds are dispersed by the wind (Carvalho, 1994). Its mating system is not known, but the congeneric tree *D. miscolobium*, from the Brazilian Cerrado exhibits

outcrossing with an apparent self-incompatibility system (Gibbs and Sassaki, 1998), which possibly suggests a similar mating system in *D. nigra.*

Nineteen populations of *D. nigra* were sampled, covering most of its current range in the Atlantic Forest (13°–23°S and 39°–45°W), at altitudes ranging from 58 to 870 m (Figure 1; Table 1). The populations were located in four Brazilian States: Bahia, Minas Gerais, Espirito Santo and Rio de Janeiro. The distance among the *D. nigra* populations sampled in this study ranged from approximately 8 km (SCR and DIO) to 1166 km (AMA and AIA) (Figure 1). Two populations (RDO and VRD) were located in large, well-preserved biological reserves (36 000 and 22 000 ha, respectively), and one (SCR) was in a small private reserve (51 ha). The other populations were located in small, disturbed forest fragments along highways or inside farms and covered areas smaller than 10 ha, except for population DIO (40 ha). Leaves were collected from about 10 adult trees in each population, totalling 185 individuals. These trees were 8–25 m tall. To ensure adequate coverage of each studied population, random samples were taken from trees at a 10- to 600-m distance from one another across each population. Following collection, the leaves were shipped in ice from the field to the laboratory and were thereafter stored at −70 °C until the DNA extraction was performed.

DNA amplification and sequencing
Total DNA was extracted from the ground leaves and stored at −20 °C until further analyses. The screening for cpDNA amplification and polymorphism in *D. nigra* was conducted on a subset of individuals using 15 universal primer pairs for non-coding cpDNA regions previously described: *trn*L-*trn*F, *trn*L intron, *trn*T-*trn*L (Taberlet *et al.*, 1991); *trn*V-*trn*M, *psa*A-*trn*S, *trn*C-*trn*D, *trn*H-*trn*K, *trn*K1-*trn*K2, *psb*C-*trn*S (Demesure *et al.*, 1995); *trn*H-*psb*A, *psb*B-*psb*F, *rpl*20-*rps*12, *trn*S-*trn*G (Hamilton, 1999); *trn*Q-*trn*S (Grivet *et al.*, 2001); *ccmp4* L-*atp*H (Weising and Gardner, 1999). Two non-coding cpDNA regions, the *trn*V-*trn*M intergenic spacer and *trn*L intron, revealed polymorphisms in the analysed individuals and were therefore selected for a large-scale survey of haplotype variation in *D. nigra*.

Polymerase chain reaction (PCR) was performed in a 25-μl total volume containing 10–20 ng of genomic DNA, 1X Taq buffer with 2.0 mM MgCl$_2$, 0.2 ng of BSA (bovine serum albumin), 200 μM of each dNTP, 0.5 μM of each primer, 1U Taq DNA polymerase (Phoneutria, Belo Horizonte, Brazil) and autoclaved deionized water. The amplifications were carried out in an Eppendorf thermocycler with an initial step at 94 °C for 4 min (*trn*V-*trn*M) or 2 min (*trn*L), followed by 35 cycles at 94 °C for 1 min, 59 °C (*trn*V-*trn*M) or 56 °C (*trn*L) for 1 min, and 72 °C for 2 min (*trn*V-*trn*M) or 1 min (*trn*L), plus a final extension at 72 °C for 7 min. Following the PCR, the products were viewed on 1% TAE agarose gels and purified by 20% PEG (polyethylene glycol) precipitation. Each PCR product was then double-strand sequenced using the DYEnamic ET dye terminator sequencing kit (GE Healthcare, Buckinghamshire, England, UK). Sequencing reactions were analysed on a MegaBACE 1000 automated sequencer (GE Healthcare). To determine any PCR errors or sequencing artefacts, two or more independent PCR amplifications were sequenced for samples that exhibited an unexpected polymorphism.

Table 1 Location of sampled populations of *Dalbergia nigra* in the Brazilian Atlantic Forest and molecular diversity indexes

Geographic origin (abbreviated sample site)	Latitude–Longitude (S–W)	Altitude (m)	Sample size	No. of haplotypes	Haplotype diversity	Nucleotide diversity	Haplotypic richness[a]
Amargosa, Bahia State (AMA)	13°02′00″–39°36′00″	390	10	2	0.356	0.00027	0.778
Apuarema, Bahia State (APU)	13°51′00″–39°44′00″	296	8	3	0.464	0.00039	1.250
Barro Preto, Bahia State (BPR)	14°46′10″–39°32′37″	544	10	2	0.356	0.00027	0.778
Vitória da Conquista, Bahia State (VCO)	15°03′11″–40°44′52″	644	11	2	0.327	0.00025	0.727
Itapebi, Bahia State (ITA)	16°00′08″–39°35′10″	144	10	1	0.000	0.00000	0.000
Eunapólis, Bahia State (EUN)	16°19′11″–39°35′50″	186	6	1	0.000	0.00000	0.000
Itamaraju, Bahia State (MAR)	16°53′49″–39°27′16″	81	10	2	0.356	0.00027	0.778
Medina, Minas Gerais State (MED)	16°13′00″–41°29′00″	587	10	3	0.511	0.00051	1.278
Padre Paraíso, Minas Gerais State (PPA)	17°06′00″–41°29′00″	780	14	4	0.736	0.00073	1.973
Teofilo Otoni, Minas Gerais State (TOT)	17°51′00″–41°30′00″	334	10	3	0.378	0.00043	1.000
Rio Doce State Park, Marliéria, Minas Gerais State (RDO)	19°42′00″–42°30′36″	262	10	4	0.733	0.00084	2.052
Santa Cruz Reserve, Dionisio, Minas Gerais State (SCR)	19°48′00″–42°43′00″	410	10	2	0.200	0.00031	0.500
Dionísio, Minas Gerais State (DIO)	19°52′00″–42°46′00″	330	10	1	0.000	0.00000	0.000
Abre Campo, Minas Gerais State (ACA)	20°20′04″–42°27′06″	680	10	2	0.467	0.00036	0.917
Pará de Minas, Minas Gerais State (PMI)	19°53′51″–44°33′43″	870	10	2	0.200	0.00015	0.500
Vale do Rio Doce Reserve, Linhares, Espirito Santo State (VRD)	19°09′03″–40°00′12″	58	9	5	0.722	0.00081	2.222
Venda Nova do Imigrante, Espirito Santo State (VNI)	20°18′59″–41°07′02″	790	10	3	0.378	0.00031	1.000
Santa Maria Madalena, Rio de Janeiro State (SMA)	22°03′14″–41°56′26″	630	5	1	0.000	0.00000	0.000
Itatiaia, Rio de Janeiro State (AIA)	22°27′40″–44°36′07″	860	12	2	0.303	0.00023	0.682

[a]Haplotypic richness with rarefaction equal to five.

The quality of the cpDNA sequences was checked and overlapping fragments were assembled using PHRED v. 0.20425 (Ewing and Green, 1998; Ewing *et al.*, 1998), PHRAP v. 0.990319 (http://www.phrap.org/) and CONSED 12.0 (Gordon *et al.*, 1998) to produce high quality consensus sequences. These sequences were aligned using CLUSTAL-W (Thompson *et al.*, 1994) implemented in the MEGA 3 program (Kumar *et al.*, 2004), with default gap penalties and manual verification. The *trn*V-*trn*M and intron *trn*L sequences were deposited in GenBank under accession numbers GU816029-GU816038 and GU816025-GU816028, respectively. All analyses in this study were performed using the combined data from the cpDNA sequences.

Population and phylogeographical analyses

The cpDNA haplotypes were defined by analysing sequences with DNASP 4.01 (Rozas *et al.*, 2003). The haplotype diversity (*h*) and nucleotide diversity (π) were estimated in ARLEQUIN 3.01 (Excoffier *et al.*, 2005). The allelic (haplotypic) richness (*Hr*) was calculated using the rarefaction method, standardized for the lower sample size, implemented in CONTRIB 1.02 (Petit *et al.*, 1998) (software available at http://www.pierroton.inra.fr/ genetics/labo/Software/Contrib/). The paired *t*-test for independent samples implemented in BioEstat 5.0 (Ayres *et al.*, 2007) was used to compare the genetic diversity (*h*, π and *Hr*) of small fragments with large reserve areas. The contributions of each population to the total haplotypic diversity (*CT*) and total haplotypic richness (*CTR*) were estimated using CONTRIB and partitioned into two components, the contribution due to a population's own level of diversity (*CS* and *CRS*), and its differentiation from other populations (*CD* and *CRD*), respectively (Petit *et al.*, 1998). To investigate the coefficient of genetic differentiation across all populations, two analyses were performed in HAPSTEP (update 2001; Pons and Petit,

1996): the G_{ST} value based only on the haplotype frequencies and the N_{ST} value influenced by both the haplotype frequencies and distances among haplotypes (number of mutational steps). The existence of phylogeographic structure is suggested when N_{ST} is significantly higher than G_{ST} (Pons and Petit, 1996). The phylogenetic relationships among the haplotypes were estimated using the median-joining algorithm implemented in NETWORK 4.1 (Bandelt *et al.*, 1999).

An analysis of molecular variance (AMOVA) using the pairwise difference was performed in ARLEQUIN to investigate the population structure (F_{ST} values) in *D. nigra*. To define alternative patterns of population subdivision and to estimate possible barriers to gene flow, we used, respectively, a spatial analysis of molecular variance (SAMOVA) based on the pairwise difference, performed in SAMOVA 1.0 (Dupanloup *et al.*, 2002), and the Monmonier maximum difference algorithm (Monmonier, 1973), performed in BARRIER 2.2 (Manni and Guérard, 2004). Using a simulated annealing approach, the SAMOVA program identifies groups of populations (*k*, which ranged from two to eight) that are geographically homogenous and maximally differentiated from each other (higher F_{CT} values) with 1000 permutations. By contrast, the BARRIER program implements Monmonier's algorithm, which identifies possible genetic barriers among the most differentiated groups of populations, spatially clustered, creating a Delaunay triangulation network to connect adjacent populations and, consequently, a Voronoi tessellation set (Manni *et al.*, 2004).

A multiple regression analysis (Smouse *et al.*, 1986) was performed using 10 000 permutations in FSTAT version 2.9.3.2 (Goudet, 2002). This analysis tests the effect of the independent variables, linear geographic distance between population pairs and their geographic location, on the average corrected genetic distances among individuals from population pairs. The first

variable reflects isolation by distance, whereas the second suggests vicariance events as the principal process of genetic divergence. The linear geographic distance matrix was inferred in kilometres separating population pairs, whereas the geographical location matrix was constructed by assigning a value of zero to population pairs within the same group (as defined in SAMOVA) and one to those in different groups. The genetic distances between the population pairs were estimated under the Kimura-2 parameter model using MEGA.

Demographic analyses and divergence time estimates

The hypothesis of population expansion was tested using different approaches. The pairwise mismatch distribution (Rogers and Harpending, 1992) was estimated in ARLEQUIN. Two groups of neutrality tests were computed in DNASP and ARLEQUIN: (1) Tajima's D (Tajima, 1989), Fu and Li's (1993) F^* and D^* considering the frequency of mutation (segregating sites); and (2) Fu's (1997) F_S based on the haplotype distribution. The demographic history of a population could be inferred by comparing such neutrality tests, given that a range expansion is suggested when Tajima's D and Fu's F_S are significantly negative, but F^* and D^* are not (Fu, 1997). The maximum likelihood coalescence method implemented in LAMARC 2.1.2 (Kuhner, 2006) was used to estimate the population growth rate (g) under the exponential growth model. The analysis was conducted using the Markov Chain Monte Carlo (MCMC) sampling algorithm with 10 initial chains with 10 000 sampled genealogies each and two final chains with 200 000 sampled genealogies each.

The isolation with migration (IM) coalescent model, as implemented in the program IM (update 2007; Hey and Nielsen, 2004), was used to estimate the divergence times (t) among the groups of populations of *D. nigra* previously defined by SAMOVA. Moreover, the program IM also estimated the following parameters: two migration rate (m_1 and m_2) parameters and the effective population sizes of the current (θ_1 and θ_2) and ancestral populations (θ_A). Several runs of IM were first performed to determine the appropriate previous values that captured completely the posterior distributions of the parameters. Subsequently, the runs were processed to verify the convergence of the selected previous parameters with different random seed numbers, as assessed by the autocorrelation of the parameters lower than 0.01, effective sample sizes (ESS) larger than 100 and update rates higher than 2%. The previous values used for the final analyses were as follows: $t = 15$, $\theta_1 = \theta_2 = \theta_A = 15$ and $m_1 = m_2 = 5$. Two final runs were conducted with four Metropolis-coupled MCMC, each run for 20 million steps after a burn-in period of 5 million steps. The MCMC approach assumed the HKY model of sequence evolution and an inheritance scalar of 0.5 for the maternally inherited cpDNA. The time units were converted to an estimate of the absolute time, T (time in years), using $T = t/uk$, where u is the number of substitutions per site per year and k is the sequence length of the cpDNA region under study. Considering that a substitution rate had not yet been estimated for the cpDNA genome of *Dalbergia*, we used the cpDNA synonymous substitution rate of 1.01×10^{-9} per site per year found in seed plants (Graur and Li, 1999).

In addition to the divergence time estimated using the coalescent-based approach, the timing of the divergences among the groups of populations was also estimated as $T = d_A/2\mu$, where d_A was the net pairwise divergence per base pair, as obtained in MEGA 3 under the Kimura-2 model, and μ was the nucleotide substitution rate per site (Nei and Kumar, 2000). The substitution rate was the same as in the analyses outlined above. In addition to reporting the point estimate for d_A, a conservative range for divergence time was calculated by applying the 95% confidence interval (CI ± 1.98 s.e.).

Results

Diversity and phylogeographic structure

A total of 1297 bp from two cpDNA regions (*trn*V-*trn*M intergenic spacer—717 bp, and *trn*L intron—580 bp) of 185 individuals of *D. nigra* were sequenced. The molecular diversity indexes are shown in Table 1. These cpDNA sequences represented 15 haplotypes with 11 polymorphic sites, including one transition, nine transversions and only one indel (Table 2). The higher number of transversions than transitions observed in the analysed sequences was expected because the cpDNA regions have a higher AT content, and thus a significant tendency towards transversions in the nucleotides flanked by A and/or T (Kelchner, 2000).

The haplotype diversity (h) for each population ranged from 0 to 0.736, the nucleotide diversity (π) from 0 to 0.00084 and the haplotypic richness (Hr) from 0 to 2.222 (Table 1). The total haplotype and nucleotide diversities were 0.7525 (s.d. ± 0.0211) and 0.00089 (s.d. ± 0.00064), respectively. Four of the 19 analysed populations were monomorphic (ITA, EUN, DIO and SMA), whereas the remaining populations showed two to five haplotypes (Tables 1 and 2; Figure 2). Haplotypes H2, H6, H13 and H15 were unique to populations AMA, MED, VNI and AIA, respectively (Table 2).

The populations with the highest diversities as evaluated by h, π and Hr were those from large biological reserves (VRD and RDO) and population PPA, located in one small, disturbed fragment on a highway edge in northeastern Minas Gerais State (Table 1). The *D. nigra* populations contributed differently to the total genetic diversity and differentiation inferred for this species. The contributions from the diversity (CS and CRS) and differentiation (CD and CRD) components of each *D. nigra* population are shown in Figure 3. The highest CS and CRS values were identified in populations RDO, VRD and PPA due to their higher h and Hr values (Table 1; Figure 3). By contrast, the highest CD and CRD were seen in populations SMA, AIA and MED (Figure 3). These contributions to genetic differentiation were probably due to exclusive mutations found in medium to high frequencies in these three populations (see haplotypes H6, H14 and H15 in Table 2). The comparison of the genetic diversities of populations from small fragments (TOT, SCR, DIO, ACA, PMI and VNI) with those from large reserve areas (RDO and VRD; Figure 2), both pertaining to same geographical group (see below the results of SAMOVA program), showed significantly lower genetic diversities in all estimates in the small fragments, such as h ($t = 6.57$, $P < 0.001$), π ($t = 8.57$, $P < 0.001$) and Hr ($t = 8.13$, $P < 0.001$).

The AMOVA analysis showed a very strong differentiation among all *D. nigra* populations ($F_{ST} = 0.624$, $P < 0.0001$). In the SAMOVA analysis, the best grouping scheme divided the distribution range of *D. nigra* into three main geographical groups ($k = 3$ and $F_{CT} = 0.668$). When $k = 2$ or $k \geqslant 4$, the SAMOVA showed similar or lower F_{CT} values. Furthermore, the grouping structure disappeared when $k \geqslant 4$, meaning that the fourth and following groups comprised only one population. Considering these results, the Monmonier algorithm (BARRIER program) suggested the following main barriers to gene flow in the *D. nigra* distribution range: (1) one barrier located approximately in the northeast of Minas Gerais and south of Bahia, dividing the natural distribution of this species into northern and southern groups, and (2) another barrier located between Espirito Santo and Rio de Janeiro, thus isolating the *D. nigra* populations located in the extreme south of the sampling area (Figure 2). The northern group (NG) is composed of populations from Bahia and northeast Minas Gerais (13°–18°S). The southern group (SG1) is formed by populations from Espirito Santo and central and south Minas Gerais (18°–21°S). The extreme southern group (SG2) includes only populations from Rio de Janeiro (22°–23°S).

The multiple regression analysis indicated that approximately 69% of the variation (R-squared) in the genetic distances could be predicted by the geographical location (partial correlation coefficient = 0.463, $P < 0.01$) and the linear geographical distance among locality pairs (partial correlation coefficient = 0.689, $P < 0.01$). This result indicates that both vicariance processes and isolation by distance explain the present-day genetic structure of *D. nigra*. The N_{ST} (0.649 ± 0.0624; $P < 0.05$) was significantly higher than the G_{ST} (0.559 ± 0.0690), indicating a phylogeographic structure, that is, closely related haplotypes in the *D. nigra* populations were found more often in the same area than less closely related haplotypes.

The phylogenetic relationships among the haplotypes were resolved by a network analysis (Figure 2). Haplotypes H1 to H6, H7 to H13 and H14 to H15 were restricted to the NG, SG1 and SG2 groups, respectively, except haplotype H7, which occurred in four individuals from population PPA of the NG group (Figure 2; Table 2). Considering that several haplotypes were connected by one mutation to one of the two most common and widespread haplotypes (H1 and H7), this star-shaped network could indicate a relatively recent population expansion in the *D. nigra*. Independent mutation events converging to a shared haplotype could explain some of the connections observed in the network analysis.

Demographic history and divergence time

The historical demography was analysed on the populations groups suggested by SAMOVA. The Fu's F_S tests performed on the *D. nigra* populations were significantly negative for all populations considered together ($P < 0.01$) and for each group considered separately ($P < 0.05$), except SG2 (Table 3). According to Fu (1997), using a 5% cutoff in F_S test will increase possibility of rejecting the null hypothesis (neutrality-demographic stability) when it is true. Thus, a clear signal of demographic expansion by Fu's F_S test was revealed only for all populations together. Tajima's D tests were

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Table 2 Variable sites of aligned sequences of two cpDNA regions (*trnV-trnM* and intron *trnL*) from which 15 haplotypes of *Dalbergia nigra* were identified

Haplotype	trnV-trnM 075	115	116	132	345	548	617	656	trnL					AMA	APU	BPR	VCO	ITA	EUN	MAR	MED	PPA	TOT	RDO	SCR	DIO	ACA	PMI	VRD	VNI	SMA	AIA	Total
H1	T	T	T	C	T	T	A	A	A	.	A	A	.	8	6	8	9	10	6	8	2	6												63
H2	.	G	.	.	.	.	.	.	T	.	.	.	.	2																			2
H3	G	.	.	.	.	.	.	.	.	.	.	.	.		1		2																3
H4	.	.	.	.	.	T	.	.	.	.	.	T	.			2		2				3											7
H5	.	.	.	.	T	.	.	.	.	.	.	.	.						2		1												3
H6	.	.	.	.	.	.	T	.	.	.	.	.	.							7													7
H7	.	.	C	.	.	.	.	.	.	.	.	.	.									4	8	5	9	10	7	9	5	8			65
H8	.	.	C	G	.	.	.	.	.	.	.	.	.										1	2									3
H9	.	.	C	G	G	.	.	.	.	.	.	.	.										1	2				1	1				5
H10	.	.	C	.	.	.	.	.	.	-	.	.	.										1	1			3						5
H11	.	.	C	G	.	.	.	.	.	.	.	.	T												1				1				2
H12	.	.	C	G	.	.	.	.	T	.	.	.	.																1	1			2
H13	.	.	C	.	.	.	.	A	.	.	.	.	.																	1			1
H14	.	.	C	.	.	.	.	A	.	G	.	.	.																		5	10	15
H15	.	.	C	.	.	.	.	A	.	G	G	.	.																			2	2

Dashes indicate indels. Sequences are numbered from the 5′ to the 3′ end in each region. The haplotypes were distributed for each population.

States/populations: Bahia (AMA, APU, BPR, VCO, ITA, EUN, MAR, MED); Minas Gerais (PPA, TOT, RDO, SCR, DIO, ACA, PMI); Espirito Santo (VRD, VNI); Rio de Janeiro (SMA, AIA).
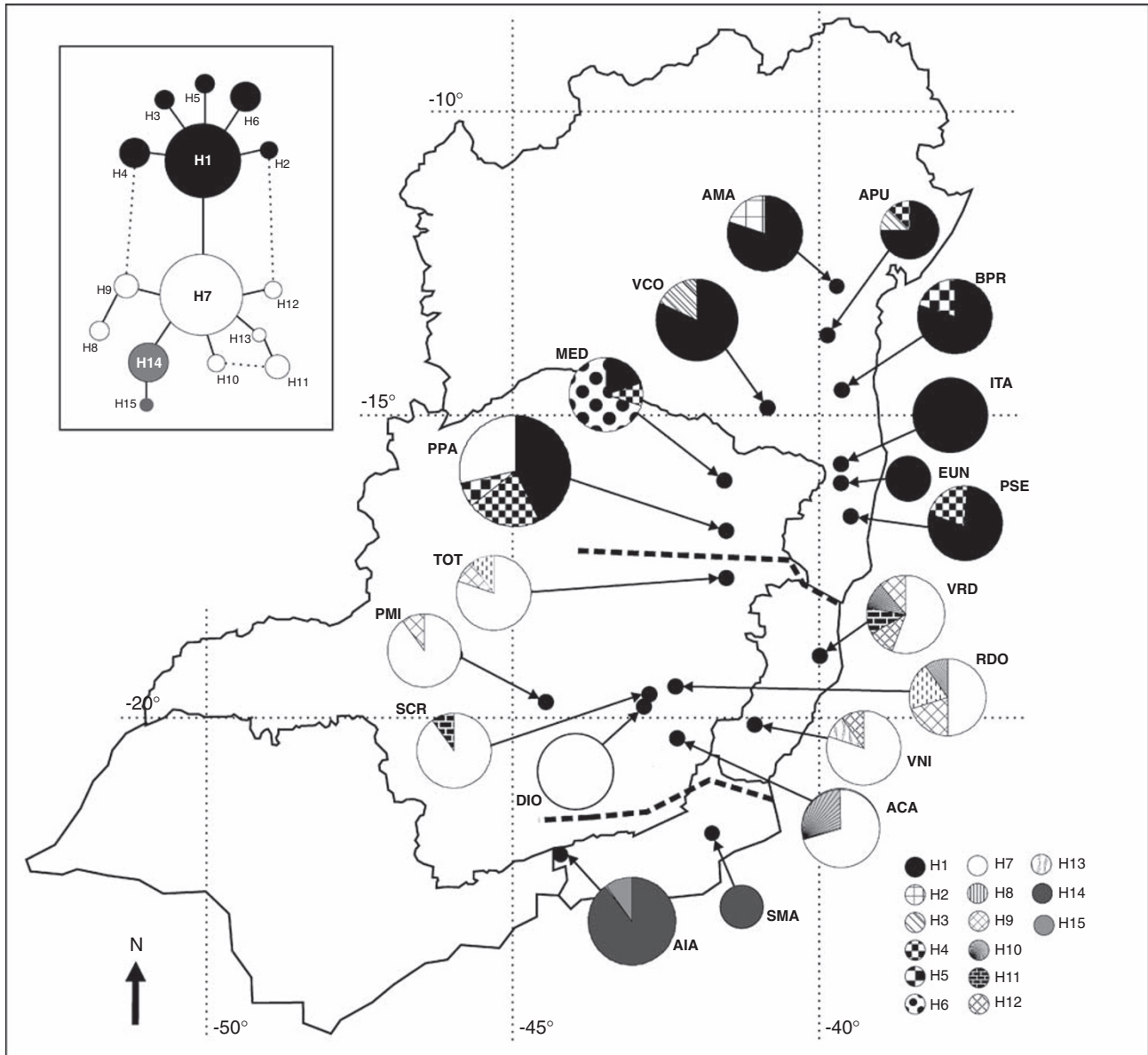
Figure 2 Map showing the geographic distribution of the cpDNA haplotypes in the *Dalbergia nigra* populations. The haplotypes are identified in the legend. The broken lines indicate the genetic barriers as defined by the Monmonier algorithm. See Table 1 for the population abbreviations and molecular diversity indexes. The median-joining network analysis is represented on the upper left. The size of each circle is proportional to that haplotype frequency across populations. Each line between the haplotypes represents a mutational step (see Table 2 for details). The dotted lines indicate independent mutation events converging on a shared haplotype. The colors of circles indicate the occurrence of these haplotypes in the three population groups (black: NG group; white: SG1 group; grey: SG2 group).

negative for all groups, although not significant, and the other neutrality tests (Fu and Li's D and F) were also not significant in any sample ($P > 0.05$) (Table 3). The mismatch distribution was unimodal taking all populations into account but not for each group taken separately (Table 3). The growth rates (average g values) were high for both NG and SG1 (Table 3), however the confidence intervals were broad and had zero overlap in some of the Bayesian runs (Table 3). The g value was not estimated for the SG2 population group due to its small sample size.

The divergence time inferred from net pairwise divergence ($d_A = 0.00071$) and substitution rate (1.01×10^{-9} per site per year) suggested that NG and SG1 diverged approximately 351 000 years before the present (ybp) (CI = −323 092–1 025 072). The time of divergence between SG1 and SG2 ($d_A = 0.00079$; T = 389 000 ybp; CI = −400 538–1 178 953) was similar to that between NG and SG1.

Using the IM coalescent model, the parameters estimated were coincident in two independent runs (Table 4), so the highest residence time (Hipt) estimates obtained in the run with the largest ESS for divergence time are described below. The divergence time between the main groups, NG and SG1, was estimated at 612 595 ybp (lower bound of HPD 90% interval: 372 137). The two groups of southern region, SG1 and SG2, diverged earlier, at 784 351 ybp (lower bound of
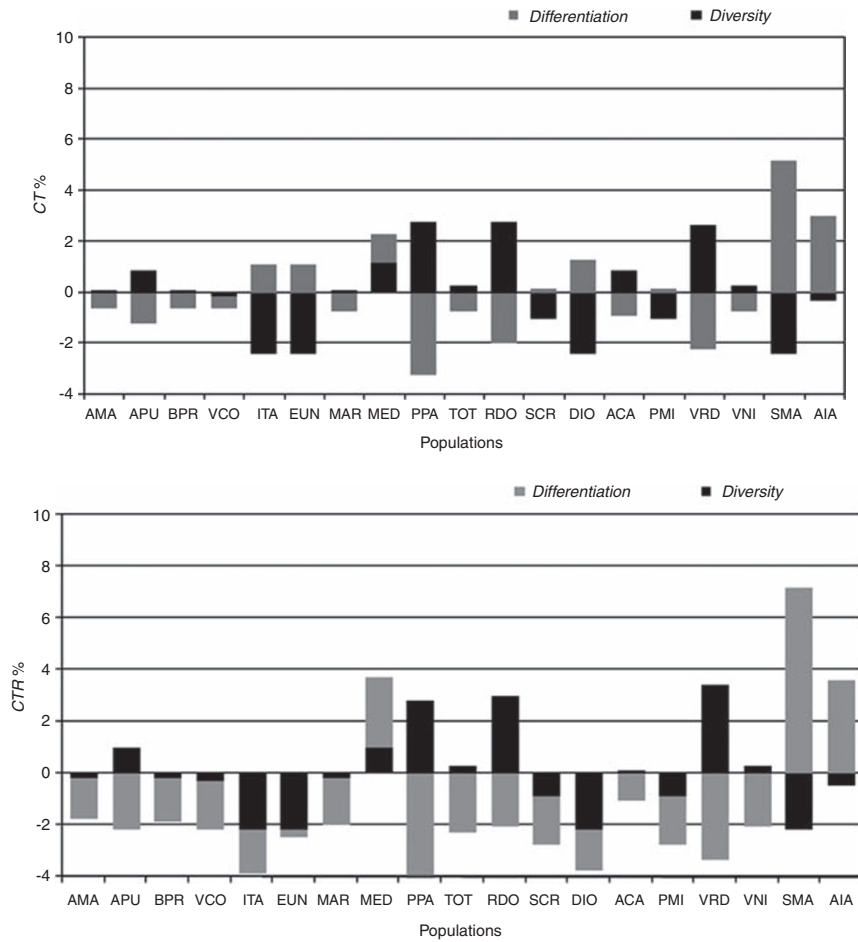
Figure 3 The contribution to the total haplotype diversity (*CT*) and haplotypic richness (*CTR*) of each population of *Dalbergia nigra* using the cpDNA haplotypes. The grey and black bars represent the contribution of diversity (*CS* and *CSR*) and differentiation (*CD* and *CDR*), respectively. See Table 1 for the population abbreviations.

Table 3 Summary statistics and demographic expansion tests observed in northern (NG) and southern (SG1 and SG2) groups and in all populations of *Dalbergia nigra*

Parameters	NG	SG1	SG2	All populations
Sample size	89	79	17	185
Number of populations	9	8	2	19
Number of variable sites	6	5	1	11
Number of haplotypes	7	7	2	15
Haplotype diversity (s.e.)	0.4872 ($\pm$0.0628)	0.3979 ($\pm$0.0685)	0.2206 ($\pm$0.1208)	0.7525 ($\pm$0.0211)
Nucleotide diversity	0.00043 ($\pm$0.00039)	0.00042 ($\pm$0.00039)	0.00017 ($\pm$0.00024)	0.00089 ($\pm$0.00064)
Haplotypic richness[a]	3.448	2.935	1.000	—
Tajima's D	−1.216	−1.092	−0.491	−0.878
Fu and Li's (1993) F'	0.418	0.364	0.426	0.639
Fu and Li's (1993) D'	1.129	0.962	0.677	1.335
Fu's (1997) F_S	−3.482*	−3.698*	0.035	−7.427**
Mismatch distribution	non-unimodal	non-unimodal	non-unimodal	unimodal
Population growth (*g*)	873.35	865.96	—	935.35
95% CI of *g* parameter	(−442.02, 993.33)	(−459.69, 985.16)	—	(−338.80, 1,121.14)

[a]Haplotypic richness with rarefaction equal to 17. *$P<0.05$, **$P<0.01$.

HPD 90% interval: 761 450). Unlike all of the other estimates, the divergence time exhibited in all runs had update rates lower or equal to 1% and posterior distribution was always flat, that is, non-zero at the upper end, suggesting that these results should be taken with caution. The effective population size estimated was twice as large in the NG group (θ_{NG}: 2.1165; HPD 90% interval: 0.6423–4.7699) than in the SG1 group (θ_{SG1}: 1.0424; 0.2422–3.0009), whereas both were higher than the ancestral population size (θ_A: 0.3264; lower bound of HPD 90% interval: 0.0105), revealing a population expansion after the split of the NG and SG1 groups.

54

Table 4 Results of two independent runs evaluating northern (NG) and southern (SG1 and SG2) groups of *Dalbergia nigra* under an isolation with migration model

Pairwise analysis	Estimate	Population size			Migration		Time	
		θ_{NG}	θ_{SG1}	θ_A	m_{NG-SG1}	m_{SG1-NG}	t	T (Ca. Year)
NG-SG1	Hipt	1.9690	1.0635	0.0105	0.4825	0.0025	0.8325	635 496
	HPD90Lo	0.6002	0.2422	0.0105	0.0025	0.0025	0.3825	291 984
	HPD90Hi	4.7067	3.1062	—	2.0275	1.2525	—	—
	Hipt	2.1165	1.0424	0.3264	0.4575	0.0025	0.8025	612 595
	HPD90Lo	0.6423	0.2422	0.0105	0.0025	0.0025	0.4875	372 137
	HPD90Hi	4.7699	3.0009	—	2.0175	1.2925	—	—
		θ_{SG1}	θ_{SG2}	θ_A	$m_{SG1-SG2}$	$m_{SG2-SG1}$	t	T (Ca. Year)
SG1-SG2	Hipt	1.4281	0.1489	0.0263	0.0025	0.0950	0.9675	738 549
	HPD90Lo	0.3417	0.0263	0.0088	0.0025	0.0050	0.6225	475 190
	HPD90Hi	3.4782	1.6909	—	1.3525	5.2450	—	—
	Hipt	1.2528	0.1314	0.0263	0.0025	0.1225	1.0275	784 351
	HPD90Lo	0.3066	0.0263	0.0088	0.0025	0.0025	0.9975	761 450
	HPD90Hi	3.5307	1.7084	—	1.3775	3.5725	—	—

Parameter estimates: θ, effective population sizes of current (θ_{NG}, θ_{SG1} and θ_{SG2}) and ancestral populations (θ_A); m, migration rate between NG-SG1 and SG1-SG2; t, divergence time between the groups of populations; T, divergence time in years.
Results according to Hey and Nielsen (2004): Hipt, highest residence time (Hipt) of posterior distribution; HPD90Lo, lower bound of the estimated 90% highest posterior density (HPD) interval; HPD90Hi, upper bound of the estimated 90% HPD interval; (−) HPD90Hi not showed when the posterior distribution was flat, that is, non-zero at the upper end.

The analysis between SG1 and SG2 showed a similar SG1 effective population size (θ_{SG1}: 1.2528; 0.3066–3.5307) to that obtained through the comparison between NG and SG1, indicating the robustness of the analysis. The effective population size was much lower in the SG2 (θ_{SG2}: 0.1314; 0.0263–1.7084) and the ancestral population to SG1 and SG2 (θ_A: 0.0263; lower bound of 90% HPD: 0.0088) than in the SG1 group. However, though the θ_A estimates exhibited high update rates and ESS, the posterior distributions were flat. The migration rate from NG to SG was 0.4575 (0.0025–2.0175) and the reciprocal migration was very low (0.0025; 0.0025–1.2925). The migration rate estimates between SG1 to SG2 were also low (0.0025; 0.0025–1.3775) whereas the migration from SG2 to SG1 was higher (0.1225; 0.0025–3.5725) (Table 4).

Discussion

Our analyses of the cpDNA sequences from *D. nigra* populations indicated a high level of genetic structure, with 62% of the total genetic diversity due to differences among populations ($F_{ST} = 0.624$), identified as three phylogeographical groups, the northern (NG), southern 1 (SG1) and southern 2 (SG2) groups (Figure 2). *D. nigra* is more genetically structured than *Eugenia uniflora* ($F_{ST} = 0.21$), an Atlantic Forest tree previously analysed by cpDNA PCR–RFLP (Salgueiro et al., 2004). However, the genetic structure of *D. nigra* is weaker than that of *Caesalpinia echinata* ($\Phi_{ST} = 0.911$; Lira et al., 2003), based on chloroplast microsatellites, but similar to *Hymenaea courbaril*, based on cpDNA sequences ($F_{ST} = 0.604$; Ramos et al., 2009), both of which are leguminous tree species found in the Atlantic Forest. The genetic split separating the NG and SG1 groups of *D. nigra* was found around 18°S latitude, between the Jequitinhonha and Doce rivers, coinciding with the split zone range found in several animal species (Costa, 2003; Pellegrino et al., 2005; Moraes-Barros et al., 2006; Cabanne et al., 2007; Tchaicka et al., 2007). The other genetic disjunction in

D. nigra, between the SG1 and SG2 groups, was located around 21°S, in the same region of separation between genetic groups found in another two legume trees, *Caesalpinia echinata* (Lira et al., 2003) and *Hymenaea courbaril* (Ramos et al., 2009). However, in the *Podocarpus* species (Ledru et al., 2007), the separation among the three groups appears not to coincide with the split zones found in legume trees. These latitudinal genetic disjunctions found in different groups of species point to historical vicariance events in the central Atlantic Forest. This scenario is corroborated by our regression analysis, which indicated that a major part of the diversity of *D. nigra* is due to these vicariance events (partial correlation coefficient = 0.463, $P < 0.01$).

Considering only the genetic divergences among the groups, the estimated divergence times between the NG and SG1 groups and between the SG1 and SG2 groups were similar (351 000 and 389 000 ybp, respectively). We applied the IM analysis taking into account demographic events because of possible differences in the effective population sizes between populations and gene flow after split. This analysis showed a more ancient separation time between these groups, 612 000 ybp (NG/SG1 groups) and 784 000 ybp (SG1/SG2). In spite of the differences between the estimates with the two approaches, both are concordant in pointing to vicariance events in the Middle Pleistocene. The divergence time between the NG and SG1 groups of *D. nigra* was similar to that found between the northern and southern groups of *Cerdocyon thous*, a widely distributed South American canid species (ca. 400 000–600 000 ybp; Tchaicka et al., 2007). However, the time of split between the lineages of the same latitudinal range in other studies with animal species is variable, ranging from the Late Pleistocene (Cabanne et al., 2007) to before the Pleistocene (Costa, 2003; Pellegrino et al., 2005).

Palynological data revealed that southeastern Brazil was cooler and drier during the glacial periods than it is in the present day (Behling, 2002). During the Last

Glacial Maximum (LGM), around 27 500–14 500 ^{14}C years ago, the southern portion of the semi-deciduous forests was replaced by subtropical grasslands, which were displaced at least 750 km further north, from latitudes of 28°/27°S to about 20°S (Behling, 1998). These data showed that the drier conditions of the LGM caused the Atlantic Forest to become much smaller and more fragmented than it is today (Behling, 2002). As a result of this constriction, the rain forest was probably partly replaced by semi-deciduous forest in southeastern Brazil and by semi-deciduous forest and caatinga (tropical semi-desert shrub vegetation) in northeast Brazil (Behling, 1998, 2002). A palynological study in the region of the split region between the Doce and Jequitinhonha rivers, performed by Behling (1995) in Lago do Pires (17° 59′S, 42° 13′W) showed that this region in the early Holocene was dominated by species from Cerrado (the Brazilian savannah), suggesting the occurrence of long dry seasons. This study also suggested that the current dense and closed semi-decidous forest in this region was only established in the latest Holocene (beginning 970 years ago). The recurrence of climatic changes during the Pleistocene in the central part of the Atlantic forest, with cycles of forest expansion and contraction, likely led to repeated vicariance events and thus resulted in the genetic differentiation of the three population groups of *D. nigra*. In fact, the location of the current NG group is included in the putative Bahia refugium, a large stable region from the Doce River northward to the southern border of the São Francisco River described by Carnaval and Moritz (2008).

Our analysis showed evidence of weak population expansion in the NG and SG1 of *D. nigra*, as indicated by the star-like topology of the network, a borderline significance of neutrality deviation in Fu's F_s test, and the Tajima's D test showed a negative deviation, although not significant. The demographic parameters estimated in the IM corroborate with the hypothesis of population expansion because that effective sizes of the ancestral populations of the groups were lower than respective derivate populations, that is, after the split there were population expansions. The NG and SG1 exhibited high diversities (Table 3), whereas the southernmost group (SG2) had low diversity, as expected for a recently founded population. Furthermore, the NG and SG1 showed higher effective population sizes than the SG2. Because the effective size is a harmonic average of the effective size across generations, it can be suggested that the northernmost populations (NG and SG1) maintained large populations during a great period and that the SG2 may have undergone a more pronounced bottleneck. This interpretation is concordant with the indication that forest fragmentation was stronger in the southern part of Atlantic Forest as a consequence of past climatic oscillations (Behling, 1998). Population expansion in SG2 may have not been detected due to a low statistical power of the analysis, as a consequence of the small sample sizes coupled with very low levels of diversity in this group. The northern group's PPA population exhibited haplotypes typical of this group (H1, H4 and H5) and also the haplotype H7, the most common haplotype in the southern (SG1) group, suggesting secondary contact due to the recent migration of propagules from the latter group. The PPA population is located near the studied site by Behling

(1995); Lago Pires, where a more recent expansion of the Atlantic semidecidous forest was registered.

In addition to the signature of the effects of past climatic changes on the phylogeographic structure of *D. nigra*, our data suggest that recent human-mediated activities such as deforestation and fragmentation have also affected the genetic diversity of the populations. It is noteworthy that in the same phylogeographic group (SG1), two populations located in large biological reserves (RDO and VRD) showed significantly higher levels of cpDNA diversity than populations from small forest fragments, for all measured parameters, including the allelic richness corrected for the sample size. These results are in accordance with a previous study (Ribeiro *et al*., 2005), which suggested that anthropogenic disturbance may have decreased the allozyme genetic diversity of *D. nigra* in these fragments. Disturbances may have eliminated some haplotypes that were once present in these populations. These molecular data are evidence that timber exploration and deforestation have promoted genetic depletion in this species, and also that the present diversity may represent only a part of the genetic diversity seen in the past in this wide geographical area of the Atlantic Forest. This scenario found for *D. nigra* could probably be extended to other timber species of the Atlantic Forest.

Implications for conservation

Phylogeography and genetic diversity data can contribute to the development of effective conservation strategies. The genetic data obtained here for *D. nigra* were based on chloroplast DNA diversity. Nuclear markers could result in an alteration in phylogeographical patterns obtained here. However, considering that *D. nigra* is a threatened species from one of the global hotspots for biodiversity conservation (Myers *et al*., 2000), and taking into account the precautionary principle (Myers, 1993), we consider it important to use the cpDNA data to inform conservation procedures for this species.

The criteria for the selection of priority populations to be conserved must include both the uniqueness of a population and its diversity level, especially in terms of its allelic composition (Petit *et al*., 1998). Considering the criteria for the recognition of management units as populations with a significant divergence of allele frequencies at nuclear or organelle loci (MU; Moritz, 1994), we suggest that the three phylogeographic groups of *D. nigra* could be treated as different MUs. Therefore, significant well-preserved forest remnants containing representative genetic diversity should be identified in each of the three groups to create units of conservation. The populations RDO and VRD, located in large conservation units in the SG1 group, are among those that most contributed to the total diversity of *D. nigra*. This fact shows the importance of preserving large areas to maintain genetic diversity. These genetic data, highlighting the uniqueness of populations in each phylogeographic group must also be considered upon sampling seeds for the *ex situ* conservation of *D. nigra*.

Special attention should be given to the northeast Minas Gerais State. In a small disturbed remnant on a highway edge in this region is located the PPA population with a high genetic diversity and containing

haplotypes from the NG and SG1, with signals of current gene flow. In addition, the neighbouring population MED, also located in a small disturbed forest fragment, gave a significant contribution to the genetic diversity of *D. nigra* due to an exclusive mutation. There are no relevant conservation units in this region. The high genetic diversity and uniqueness found in these populations of *D. nigra*, plus evidence from animal studies (Ditchfield, 2000; Costa, 2003; Moraes-Barros *et al.*, 2006; Tchaicka *et al.*, 2007), indicate the high priority that should be given to implementing conservation units in northeast Minas Gerais.

Conflict of interest

The authors declare no conflict of interest.

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