Pliocene and Pleistocene events shaping the genetic diversity within the central corridor of the Brazilian Atlantic Forest

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Dinoponera lucida (Formicidae; Ponerinae) is an extinction-threatened species of ant which is endemic in the central corridor of the Atlantic Forest. We used mitochondrial sequences of the Cox1, Cox2 and Cyt*b* genes in order to infer some aspects of the evolutionary history and phylogeography of this ant. High genetic divergence and population structure were observed for the whole species. The current pattern of *D. lucida* diversity seems to be shaped during different geological times: middle Pliocene, early Pleistocene and mainly late Pleistocene, when the reduction of populations generated a structure pattern of the genetic variation of this species. Our data show that this structure results from the maintenance of populations of *D. lucida* within very small putative refuges to the south of the central *Bahia refugium*. We thus argue that, for some Atlantic forest endemic species, especially those resistant to very small fragments of forest, such as *D. lucida*, the small putative refuges were as important as, or even more important than, larger and stable refuges for the creation and maintenance of diversity, adding another piece to the puzzle of the mechanisms underlying local endemism. © 2010 The Linnean Society of London, *Biological Journal of the Linnean Society*, 2010, **101**, 949–960.

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INTRODUCTION

Ecological and historic processes of fauna diversification have been widely studied under the light of phylogeography, a relatively new discipline that deals with the spatial arrangements of genetic lineages, especially within and among closely related species (Avise, 2009). This approach was extensively used in studies regarding the fauna associated with tropical forests (Moritz *et al*., 2000), but studies involving the fauna found in the forests of the Southern Hemisphere are still rare (Beheregaray, 2008), especially those concerned with the Brazilian Atlantic Forest (Carnaval & Moritz, 2008; Carnaval *et al*., 2009).

The central corridor of such forest is one of the priority areas for Brazilian biodiversity preservation, as it presents high indices of both endemism and

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biodiversity (species richness) and also because it shelters distribution-restricted and extinction-threatened species (Aguiar *et al*., 2003; Ayres *et al*., 2005). Unfortunately, the genetic diversity and narrow endemism in this central corridor have been substantially underestimated (Carnaval *et al*., 2009).

The majority of the few reported studies concerning historical processes of fauna diversification in the central corridor of the Atlantic Forest (e.g. Cabanne *et al*., 2008; Lara-Ruiz, Chiarello & Santos, 2008; Carnaval *et al*., 2009) proposes a relation between the high indices of diversity and endemism observed and the Quaternary Refuges Model (Haffer, 1969; Brown & Ab'Saber, 1979). According to this model, population fragmentation events because of climatic fluctuation during the end of the Pleistocene would have caused a rise in diversity amongst populations once distributed in a contiguous fashion, or could even have favoured speciation events. However, some studies suggest diversification might have occurred in events prior to the Pleistocene (e.g. Lara & Patton, 2000; Fitzpatrick *et al*., 2009), indicating that one unique model of the recent climatic fluctuations cannot be taken as a general pattern and that, considering the particularities of each species, studies with different groups still have a lot to offer to the understanding of the evolution of the populations in the Brazilian Atlantic Forests.

Despite the small number of studies involving species distributed along the central corridor of the Atlantic Forest, a North–South division pattern of the populations seems to be relatively common for the species studied (e.g. sloths, Moraes-Barros *et al*., 2006; Lara-Ruiz *et al*., 2008). In some species, such as the *Gymnodactylus darwinii* lizard (Pellegrino *et al*., 2005) and the *Xiphorhynchus fuscus* bird (Cabanne *et al*., 2008), there is evidence that this North–South division pattern may have been imposed by the action of the Doce River as a barrier to the gene flow between the northern and southern regions of its estuary. To our knowledge, the only work focusing on insects restricted to the central corridor, in a biogeographic approach, studies *Dinoponera lucida* Emery, 1901, a giant ant presenting a genetic standard which is compatible with that of an ancestral population which was, in turn, divided into two isolated groups: the first to the south of the Doce River (Espírito Santo State), in which the 2n = 118 karyotype is predominant; and the second, to the north, with karyotypic variations ranging from 2n = 106 to 120 (Mariano *et al*., 2008).

Species from the *Dinoponera* Roger genus (Formicidae: Ponerinae), known as giant ants (workers reach 4 cm in length), occur exclusively in South America and present a haplodiploid sex-determination system. The genus comprises six described species with a general pattern of allopatric distribution (Kempf, 1971; Paiva & Brandão, 1995). All species within *Dinoponera* are very similar and the species diagnostic differences are rather subtle (Kempf, 1971). In this genus there are no morphologically different castes and the reproduction is performed by fertilized workers known as gamergates (Peeters, 1993). In addition, females are apterous, which has a strong impact on the dispersion and colonization abilities of the species. In contrast, males are winged and smaller than females (Kempf, 1971). *Dinoponera lucida* occurs in a limited geographical area, being restricted to the remaining fragments of the central corridor of the Atlantic Forest, which led the inclusion of this species in the list of extinction-threatened species (Campiolo & Delabie, 2008).

For this study, we used *D. lucida* individuals collected throughout the area of known occurrence of the species. Mitochondrial sequences were used in order to try to infer the evolutionary history of this ant. We decided to use only mitochondrial markers because of the colony-foundation behaviour of this species. Such behaviour occurs through colony fission and is characterized by the foundation of a new colony by the reproductive female with the help of its sterile relatives (Peeters & Ito, 2001). Hence, the population structure of this species is expected to be that highly related to maternal heritance and, since apterous females disperse less than winged males, mtDNA should keep ancient footprints that would be lost in the nuclear genome.

The data obtained were used in order: (1) to measure the genetic variability and the degree of structure in this species; (2) to test whether the division into two groups (to the north and to the south of the Doce River) is maintained when more individuals are sampled and a exclusively maternal inheritance marker is used; (3) to calculate the divergence time between demes of *D. lucida*; and (4) to infer the influence of the Pleistocene events in the evolutionary history of this ant.

MATERIAL AND METHODS

Sixty-seven *D. lucida* colonies, collected in 24 locations covering the area of occurrence of the species, were analysed (Fig. 1, Table 1). Because it is a monogynic species, it is expected that all of the individuals in the colony present the same mitochondrial inheritance and, thus, only one worker was analysed per colony.

DNA PREPARATION, AMPLIFICATION AND SEQUENCING

Total DNA was isolated from each individual using the protocol suggested by Fernandes-Salomão *et al*. (2005). A 639-bp fragment of the Cyt*b* gene and a
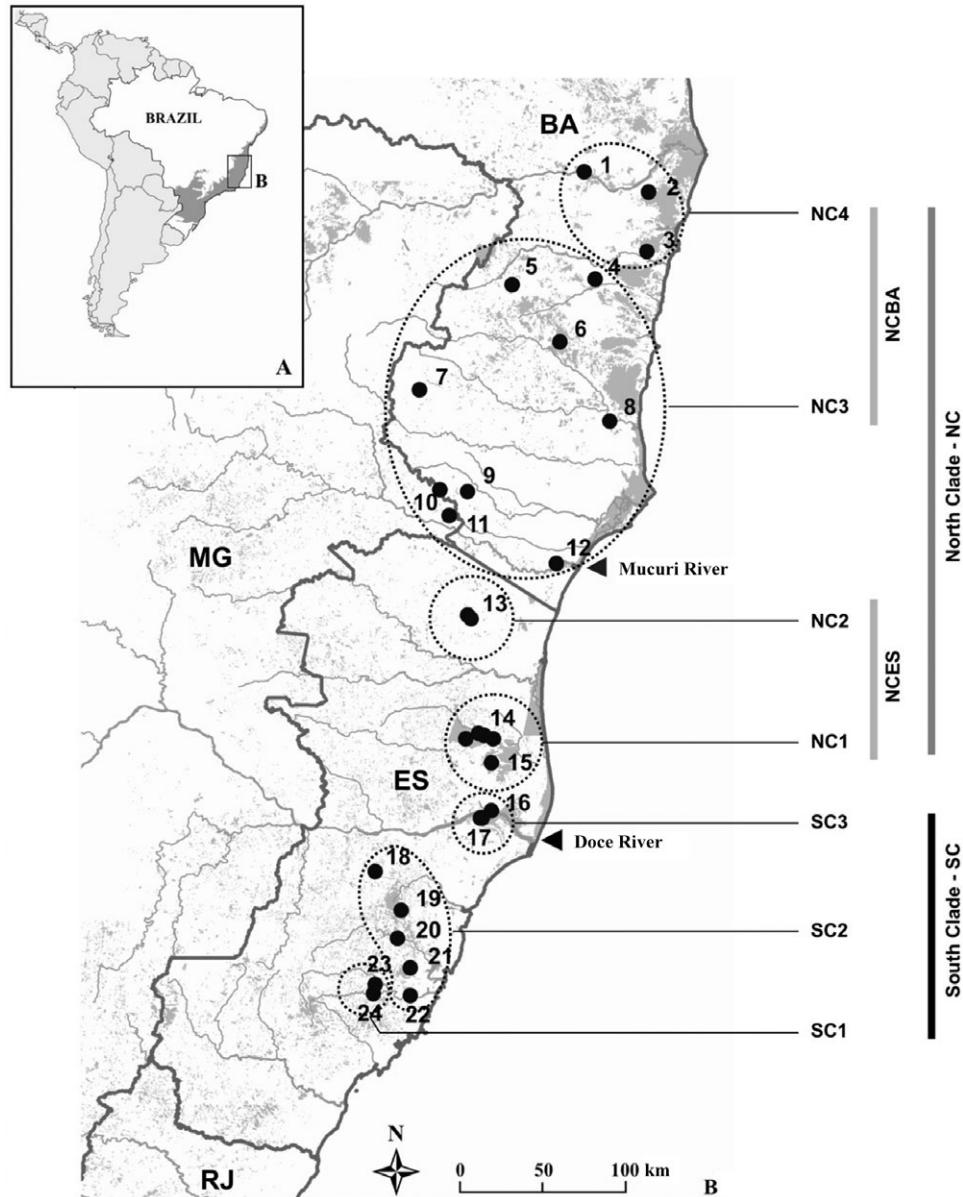
Figure 1. Area of distribution of the extinction-threatened giant ant *Dinoponera lucida* (Formicidae: Ponerinae), according to Campiolo & Delabie (2008). A, indication of the studied area on the map of Brazil, showing the original extension of the Atlantic Forest (dark grey). B, sampling points for this study. Areas in grey in the map represent the remaining fragments of the central corridor of the Atlantic Forest. The numbers refer to the sampling cities codes presented in Table 1. BA, Bahia State; ES, Espírito Santo State; MG, Minas Gerais State; NCBA, Bahia Clade; NCES, Espírito Santo Clade; RJ, Rio de Janeiro State. Vertical bars indicate the clades found in Figure 2.

573-bp fragment of the mitochondrial region including partial sequences of Cox1, tRNA-Leu and Cox2 genes were amplified by the standard PCR technique. The typical amplification conditions included a 5-min denaturation at 94 °C, followed by 35 cycles of 94 °C for 50 s, primer annealing temperature for 50 s and 72 °C for 1 min 20 s, with a final extension at 72 °C for 4 min. The primers used in this work are listed in the in the Supporting Information (Table S1). PCR products were cleaned using the Wizard SV Gel and PCR Clean-Up System (Promega). The PCR product was sequenced using dye-terminator chemistry in a MegaBace sequencer (GE Healthcare). Sequence chromatograms were evaluated and edited in the Consed program (Gordon, Abajian & Green, 1998).

Table 1. Sampled localities with respective geographic references for the sampling points and sampling number (*N*) present within the map (Fig. 1)

Sampling points	Localities	Latitude/longitude (GGMM′SS,ss″)		*N*
1	Itapebi – BA	15S57′01.67″	39W31′13.34″	1
2	Belmonte – BA	16S03′37.67″	39W10′13.33″	2
3	Porto Seguro – BA	16S22′49.68″	39W10′49.33″	3
4	Itabela – BA	16S31′49.68″	39W27′37.34″	1
5	Guaratinga – BA	16S33′37.68″	39W54′37.35″	3
6	Itamaraju – BA	16S52′13.69″	39W39′01.35″	2
7	Itanhém – BA	17S07′49.69″	40W24′37.37″	1
8	Prado – BA	17S18′01.70″	39W22′49.34″	2
9	Ibirapuã – BA	17S40′49.70″	40W09′01.37″	3
10	Lagedão – BA	17S40′13.70″	40W18′01.37″	3
11	Serra dos Aimorés – MG	17S48′37.70″	40W15′01.37″	3
12	Mucuri – BA	18S04′13.71″	39W40′13.36″	3
13	Pinheiros – ES	18S22′13.72″	40W07′49.37″	7
14	Sooretama – ES	19S01′13.73″	40W09′37.38″	4
15	Linhares/CRVD – ES	19S09′01.73″	40W01′13.38″	1
16	Linhares/INCAPER – ES	19S24′37.74″	40W04′49.38″	1
17	Linhares/F.Goytacazes – ES	19S24′37.74″	40W01′13.38″	5
18	Santa Teresa/S.A.Canaã- ES	19S44′25.74″	40W39′01.40″	3
19	Santa Teresa – ES	19S57′01.75″	40W30′3740″	2
20	Santa Leopoldina – ES	20S06′01.75″	40W31′49.40″	3
21	Cariacica – ES	20S15′37.75″	40W27′37.40″	4
22	Viana – ES	20S24′37.76″	40W27′37.40″	2
23	Domingos Martins – ES	20S21′01.75″	40W39′01.41″	4
24	Marechal Floriano – ES	20S24′37.76″	40W27′37.40″	3

BA, Bahia State; ES, Espírito Santo State; MG, Minas Gerais State.

GENETIC DATA ANALYSES

The edited sequences were initially aligned using ClustalW (Higgins *et al.*, 1994) and the alignment was corrected manually using the Mega 4.0 program (Tamura *et al.*, 2007). The tRNA-Leu fragments were excluded from the analysis and we thus concatenated the Cyt*b*, Cox1 and Cox2 sequences and produced a total alignment of 1149 bp containing 270 variable sites.

Phylogenetic relationships amongst the mtDNA haplotypes found were estimated through Bayesian Inference (Yang & Ranalla, 1997) in MrBayes 3.1 (Huelsenbeck & Ronquist, 2001; Ronquist & Huelsenbeck, 2003), using one exemplar of each haplotype. The data set was partitioned by gene and each partition was run using the MrModeltest program (Nylander, 2004) in order to identify the best partition-specific nucleotide model, inferred using MrModeltest (Nylander, 2004). For Cyt*b*, the model of choice was the generalized time reversible model (GTR) and, for Cox1 and Cox2, the Hasegawa, Kishino and Yano model (HKY) was the model chosen. Comparatively, a haplotype network was built through the median-joining network algorithm (Bandelt, Forster & Röhl, 1999) using the NETWORK ver. 4.5 software (available in http://www.fluxus-engineering.com/sharenet.htm).

In order to assess the diversity and the genetic structure of the populations, nucleotide diversity indices (π), haplotypic diversity indices (Hd) (Nei, 1987) and neutrality tests were estimated using the DnaSP software (Rozas *et al.*, 2003).

ISOLATION BY DISTANCE AND HISTORICAL PROCESSES

We used the Mantel test (Mantel, 1967) and the partial Mantel test (Smouse, Long & Sokal, 1986) to simultaneously test the effects of historical processes and isolation by distance (IBD) on the genetic structure of the species (Wright, 1943). The partial Mantel test was used to expand analyses from the simple Mantel test, incorporating multiple landscape variables (Storfer *et al.*, 2007).

The analyses were carried out with the aid of the IBD software (Bohonak, 2002) with 5000 randomizations. Three matrices were analysed: (1) genetic distance (p-distance); (2) geographic distance, calculated by the Alleles in Space (AIS) software (Miller, 2005); and (3) a binary model matrix expressing long-term

historical divergence, in which the value 1.0 indicates that two populations are linked, within the same group, and 0.0 indicates they are clustered into different groups. The groups were defined based on the groups found in the phylogenetic tree.

Given this scenario, with a partial Mantel test it would be possible to establish which part of the total explained variance of genetic distances could be attributed to (1) the effect of historical processes, (2) the effect of more recent and local IBD or (3) to the overlap between them (Telles & Diniz-Filho, 2005).

COALESCENCE ANALYSES

The Bayesian Skyline Plot (BSP) method (Drummond *et al.*, 2005), implemented by the Bayesian Evolutionary Analysis Sampling Trees (BEAST) 1.4.8 software (Drummond & Rambaut, 2007) was used to estimate the expected time to the most recent common ancestor (T_{MRCA}) for *D. lucida*. Such a method performs a Markov chain Monte Carlo (MCMC) analysis, the result of which is a posterior distribution of the effective population size through time. In order to calibrate the molecular clock, we calculated a substitution rate based on Cox1 sequences and fossil calibration of the molecular phylogeny regarding the Formicidae family (Moreau *et al.*, 2006). Both minimum and maximum divergence times for the Formicinae (92.0 ± 0.2 and 101.4 ± 3.8), Myrmecinae (99.8 ± 4.2 and 114.0 ± 4.5) and Ponerinae (110.7 ± 6.3 and 131.5 ± 5.9) subfamilies (Moreau *et al.*, 2006) were used as calibration references, providing a rate of $1.455\text{E-}02 \pm 1.25\text{E-}03$ substitutions per Myr. This rate was extrapolated for our concatenated sequences (Cox1, Cox2 and Cyt*b*).

The BEAST software had its molecular clock set to 'Relaxed Clock: Uncorrelated Log-normal' and its tree prior defined as 'Yule process'. The analyses were carried out using distinct substitution models for each codon position. We ran five independent estimatives, with 25 000 000 generations each, from which the initial 10% were burned out. The BEAST outputs trace files were analysed using TRACER ver. 1.4.1 software (Rambaut & Drummond, 2007).

Comparatively, divergence time between clades was estimated through MDIV software (Nielsen & Wakeley, 2001), which calculates the maximum likelihood estimates of three demographic parameters: theta ($\theta = 2N_{ef}\mu$), where N_e is the effective size and $\mu = 1.455\text{E-}02 \pm 1.25\text{E-}03$; (ii) M ($2m$), where 'm' is the migration rate; and (iii) T (t/N_e), where 't' is the time between two given clades since divergence. Five independent runs were executed for each divergence assessed, each with 5 000 000 generations for MCMC and burn-in time of 10%.

RESULTS

POPULATION STRUCTURE

The Bayesian consensus unrooted tree constructed with Cyt*b*/Cox1/Cox2 concatenated sequences is shown in Figure 2. The values alongside some ancestral nodes correspond to posterior probability (PP) values of the MCMC. The phylogenetic tree shows that *D. lucida* diversity may be firstly divided into two main clades (PP = 1.0), the first comprising populations sampled to the north of Doce River (North Clade; NC) and the second comprising populations sampled to the south of this river (South Clade; SC). NC is divided into four groups (NC1, NC2, NC3, NC4) with the last two groups clustered into a poorly resolved clade (PP = 0.7); while SC is divided into three groups (SC1, SC2, SC3). All groups are remarkably consistent, showing high Bayesian support (PP = 1.0, marked with an asterisk in Fig. 2). Overall, individuals from a given locality cluster together and those from near localities are more likely to cluster together than the ones from more distant localities.

Table 2 presents the results of the nucleotidic and haplotypic diversity analyses, as well as those of the neutrality tests calculated for each of the clades and subclades proposed. In spite of the fact that (1) the geographic distribution of the SC is more restricted than the one of the NC and (2) the number of samples collected for the southern region is also fewer than that for the northern region, the indices point to equivalent nucleotidic and haplotypic diversity to the north and south of the Doce River.

Despite the fact that the neutrality tests failed to detect changes in population sizes, the phylogenetic tree suggests that most populations and groups, except for NC3 and NC4, present signs of bottlenecks followed by recent expansion, characterized by long branches between groups and short branches linking the haplotypes within each group. The BSP also suggests this pattern (see below).

DIVERSIFICATION PROCESSES AND DIVERGENCE TIME

The correlation between genetic and geographic distance shows that 22% ($R^2 = 0.22$, $P < 0.0002$) of the variation in genetic distances can be attributed to the geographic distances between pairs of populations. The binary model matrix designed to separate the population groups was significantly correlated to the genetic distances matrix ($R^2 = 0.446$; $P < 0.0002$), i.e. historical processes can account for $\cong 45\%$ of the variation in genetic distances. The partial Mantel test, simultaneously associating the three matrices, showed partial correlation ($R^2 = 0.06$; $P < 0.0020$) between genetic and geographic distances, when controlling for the binary model matrix; and partial correlation
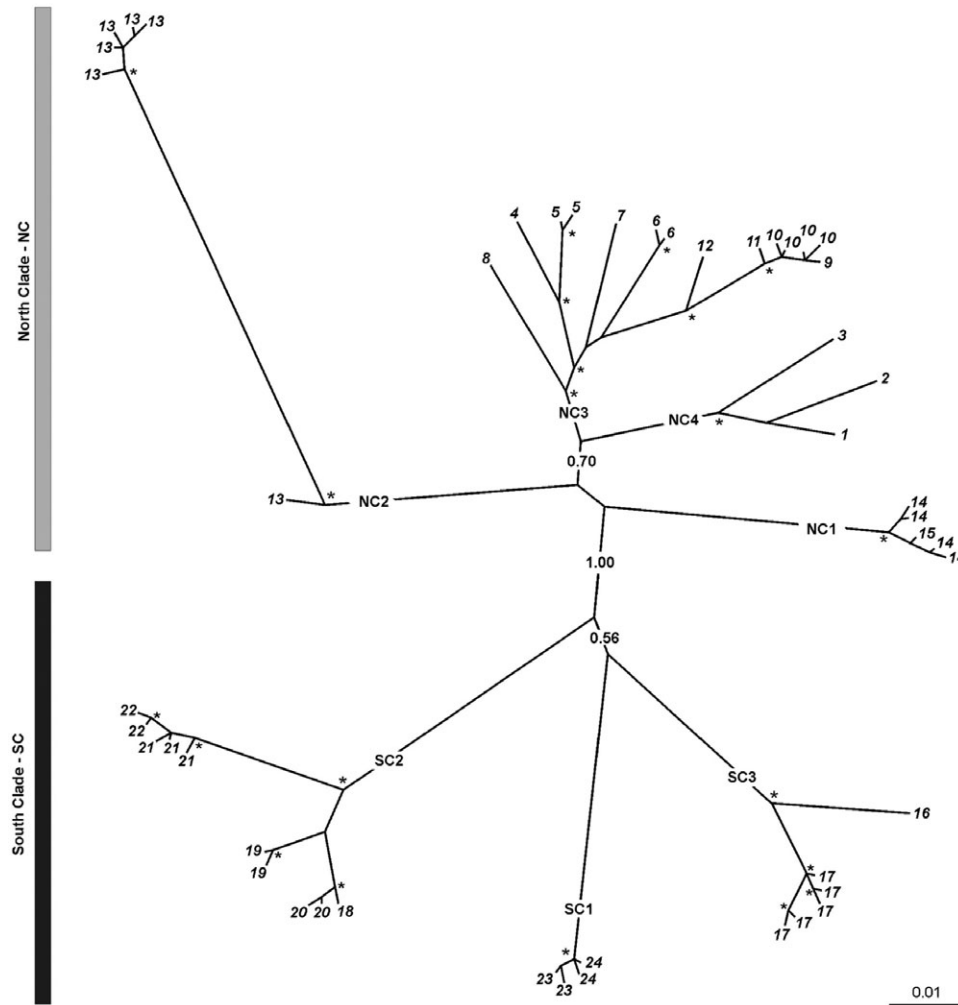
Figure 2. Consensus tree of two chains with 3 000 000 generations for Markov chain Monte Carlo (MCMC) each. Twenty-five per cent of the initial trees of each chain were eliminated. The consensus tree was reconstructed through Bayesian inference of 1149 bp of the Cox1/Cox2/Cyt*b* genes concatenated, taking into consideration distinct substitution models for each subset of *Dinoponera lucida* sequences. The *D. lucida* individuals are represented by their collection site (numbers refer to Table 1). Posterior probabilities (PP) are shown in the nodes; *PP = 1.00.

($R^2 = 0.33$; $P < 0.0002$) between genetic distance and the binary model matrix, controlling for geography.

The BSPs estimated both for the species as whole and for the North and South Clades separately are displayed in Figure 3. BSPs show that the most recent common ancestor of all samples of *D. lucida* emerged during the Pliocene (TMRCA $\cong$ 3.3 Mya, Table 3) and also that the population size was maintained until 200 000 yBP, when the whole species, as well as NC (Fig. 3B) and SC (Fig. 3C), suffered a strong reduction in effective size (N_e) (Fig. 3D, E). Regarding SC, this strong decline reaches its maximum around 25 000 yBP, when there was a slight recovery in population growth (Fig. 3F).

As NC presents groups of populations with two distinct patterns of diversification – (1) signals of bottleneck followed by recent expansions (NC1 and NC2) and (2) large branch lengths within populations, suggesting more ancient diversification (NC3 and NC4) – we decided to estimate the BSP for these two different groups separately: the Espírito Santo Clade (NCES) (NC1, NC2) and the Bahia Clade (NCBA) (NC3, NC4). Figure 4 shows these plots and highlights a population increase from 1.75 Mya to 200 000 yBP within the NCBA (Fig. 4A, C). In contrast, within the NCES, Figure 4B and D show a recent population increase, also found within SC (Fig. 3C, F).

Table 2. Nucleotidic and haplotypic diversity statistics and neutrality tests for each of the clades and subclades identified in the Bayesian tree

| | N | V | H | Hd | π | Neutrality tests | | | |
						D	D′	F′	Fs
All	67	270	48	0.989 ± 0.005	0.054 ± 0.002	−0.088	1.353	0.924	−0.785
NC	40	200	28	0.979 ± 0.010	0.043 ± 0.003	−0.076	1.092	0.800	1.319
NC1	5	6	5	1.000 ± 0.126	0.003 ± 0.001	1.241	1.241	1.286	−2.004*
NC2	7	53	6	0.952 ± 0.096	0.022 ± 0.007	0.744	1.422	1.406	1.737
NC3	22	91	14	0.952 ± 0.026	0.021 ± 0.002	−0.096	0.453	0.332	2.132
NC4	6	37	3	0.733 ± 0.155	0.016 ± 0.003	1.203	1.018	1.158	6.910
SC	27	135	20	0.974 ± 0.017	0.043 ± 0.002	1.201	0.920	1.193	1.822
SC1	7	3	4	0.810 ± 0.016	0.001 ± 0.001	−0.302	−0.519	−0.507	−1.217
SC2	14	42	10	0.945 ± 0.002	0.015 ± 0.001	1.578	1.064	1.383	1.183
SC3	6	30	6	1.000 ± 0.096	0.009 ± 0.004	−1.031	−1.007	−1.107	−0.813

N, number of sequences; V, number of variable sites; H, number of haplotypes; Hd, haplotype diversity; π, nucleotide diversity; D, Tajima's D; D′, Fu and Li's D; F′, Fu and Li's F; Fs, Fu's Fs; NC, North Clade; SC, South Clade.
*$P < 0.05$ for neutrality tests.

Table 3. T_{MRCA} – expected time to the most recent common ancestor estimated through the Bayesian Skyline Plot method implemented through BEAST 1.4.8 and the divergence time between North Clade (NC) and South Clade (SC) and between Bahia Clade (NCBA) and Espírito Santo Clade (NCES) estimated with MDIV

| BEAST – T_{MRCA} in Mya (Median values, 95% lower–upper)	
All	3.34 (2.49–4.38)
NC	2.76 (1.99–3.63)
NC1	0.15 (0.04–0.39)
NC2	1.25 (0.65–1.91)
NC3	1.50 (0.96–2.14)
NC4	1.04 (0.52–1.69)
NCBA	1.97 (1.16–3.25)
NCES	2.81 (1.93–3.95)
SC	2.78 (1.93; 3.78)
SC1	0.075 (0.019; 0.192)
SC2	0.93 (0.45; 1.50)
SC3	0.84 (0.35; 1.51)
MDIV – Divergence time (Mya)	
Divergence between NC and SC	2.47 ± 0.95
Divergence between NCBA and NCES	1.22 ± 0.96

Table 3 presents the T_{MRCA} estimated for each of the clades and subclades. The median values, confidence intervals, auto-correlation time (ACT) and effective sample size (ESS) for the BSP analyses are detailed in the Supporting Information (Table S2). The coalescence dates estimated by MDIV point to diversification since the Pliocene for the North and South Clades (2.47 ± 0.95 Mya) and to diversification since the Pleistocene for the NCBA and NCES (1.22 ± 0.96 Mya).

DISCUSSION

The current diversity pattern of *D. lucida* seems to have been shaped during different geological times, attributable to different climatic and geological events. The first of these events dates back to the Middle Pliocene (2.47 ± 0.95 Mya) and divided the populations sampled below the south margin (SC) and those sampled above the north margin (NC) of the Doce River. In fact, the phylogenetic tree shows a subdivision branch between the SC and the NC (Fig. 2), which, regardless of the relative short branch length dividing the SC and NC, as well as the low geographic distances between some clades, such as NC1 and SC3 (Fig. 1), is well supported (PP = 1).

A second division within *D. lucida* distribution dates back to the Early Pleistocene (1.22 ± 0.96 Mya) and seems to have isolated the populations sampled above the north margin of the Mucuri River (NCBA) from those sampled below the south margin of this River (NCES). Figure 1 shows that the geographic distance between populations 12 and 10 (north margin of the Mucuri River) is similar to the geographic distance between populations 12 and 13 (south margin of Mucuri river). However, the genetic distance between populations 12 and 13 (Fig. 2) is much larger. Figure 4 shows that, just after this division, NCBA increased in size, while NCES maintained its size until 200 000 yBP. Indeed, the NCBA contains the most diverse and differentiated haplotypes found in *D. lucida*. Mariano *et al*. (2008), using
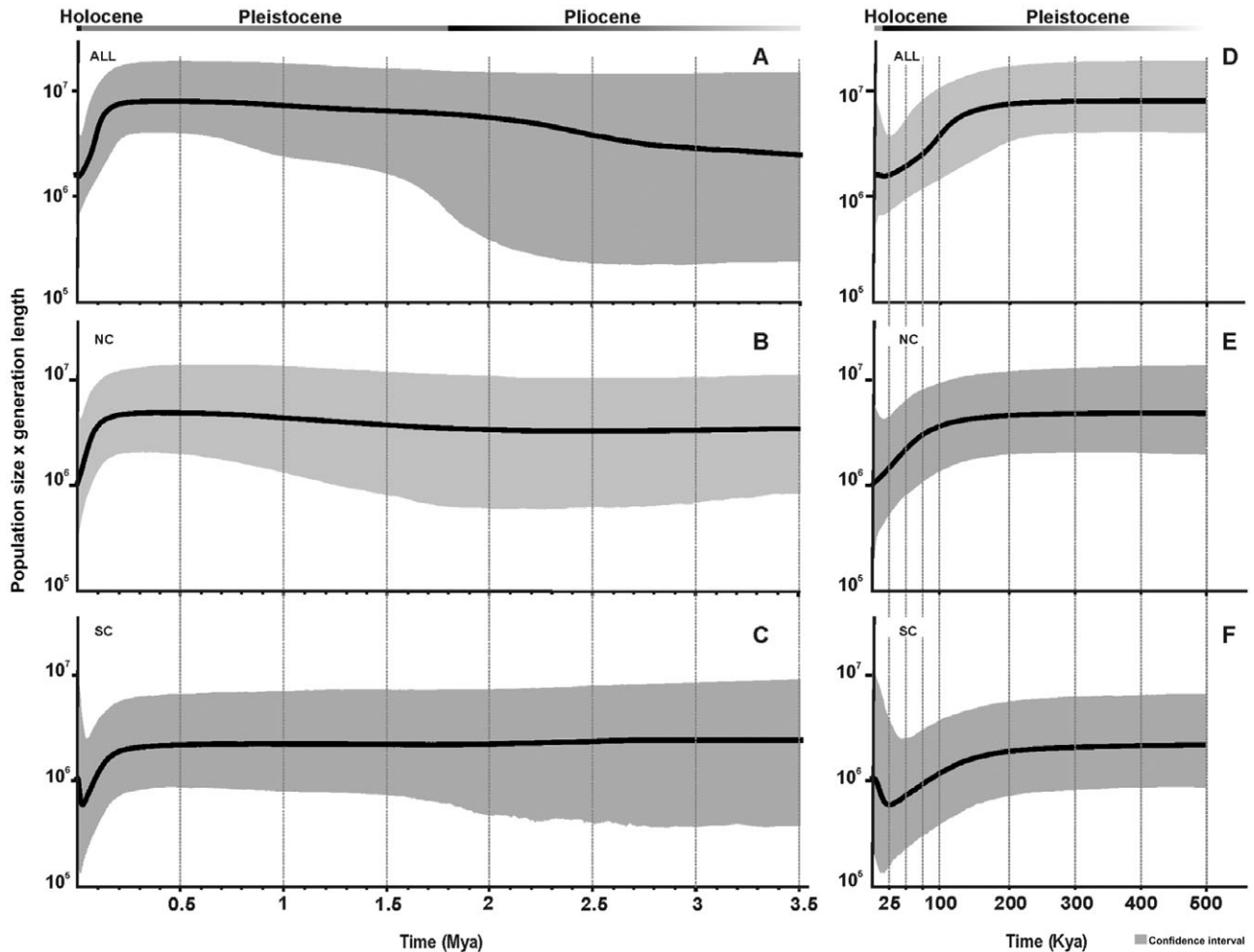
Figure 3. Bayesian Skyline Plot (BSP) implemented through BEAST 1.4.8, with 25 000 000 generations for Markov chain Monte Carlo (MCMC), burn-in time of 10% and substitution rate of 1.455E-02 ± 1.25E-03 substitutions per Myr in order to calibrate the molecular clock. A, D, BSP for all sequences. B, E, BSP for North Clade (NC). C, E, BSP for South Clade (SC), analysed separately. (D), (E) and (F) correspond to an amplified portion of the BSP, highlighting the events since the last 500 000 yBP.

cytogenetic markers in order to study different populations of *D. lucida*, also found higher diversity within populations sampled at the north margin of the Mucuri River (2N = 106–120) than within other populations (2N = 118). The Mucuri River seems to be the barrier between *Micoureus demerarae* and *Micoureus paraguayanys*. The first species, located at the north margin of the river, presents more genetic variation than the second, located at the south margin of Mucuri (André Santos Neves and Leonora Pires Costa) (Karla SC Yotoko, pers. comm.).

The third, and probably most important, event which affected the current pattern of *D. lucida* seems to have occurred during the Late Pleistocene, from 200 000 yBP, when *D. lucida* started suffering a drastic effective size reduction, probably as a result of climatic changes during this period. It is possible that

the long branch lengths found among the groups of populations shown in Figure 2 have emerged during this period, because of separated founding events. After this effective size reduction, within a fourth event, in a very recent geological time ($\cong$ 21 000 yBP), all populations below the south margin of the Mucuri River suffered a synchronic increase in N_e, which is clearly shown by the short branch lengths within these populations at the phylogenetic tree (Fig. 2) and also by the BSP of the SC (Fig. 3C, F) and NCES (Fig. 4B, D). Indeed, the southernmost clades, SC1 and SC2, although geographically very close to each other, are fairly distant genetically; i.e. our results suggest these populations, which remained isolated during the Pleistocene, accumulated differences and, today, present this pattern of differentiation, even under the absence of a current obvious geographic barrier.
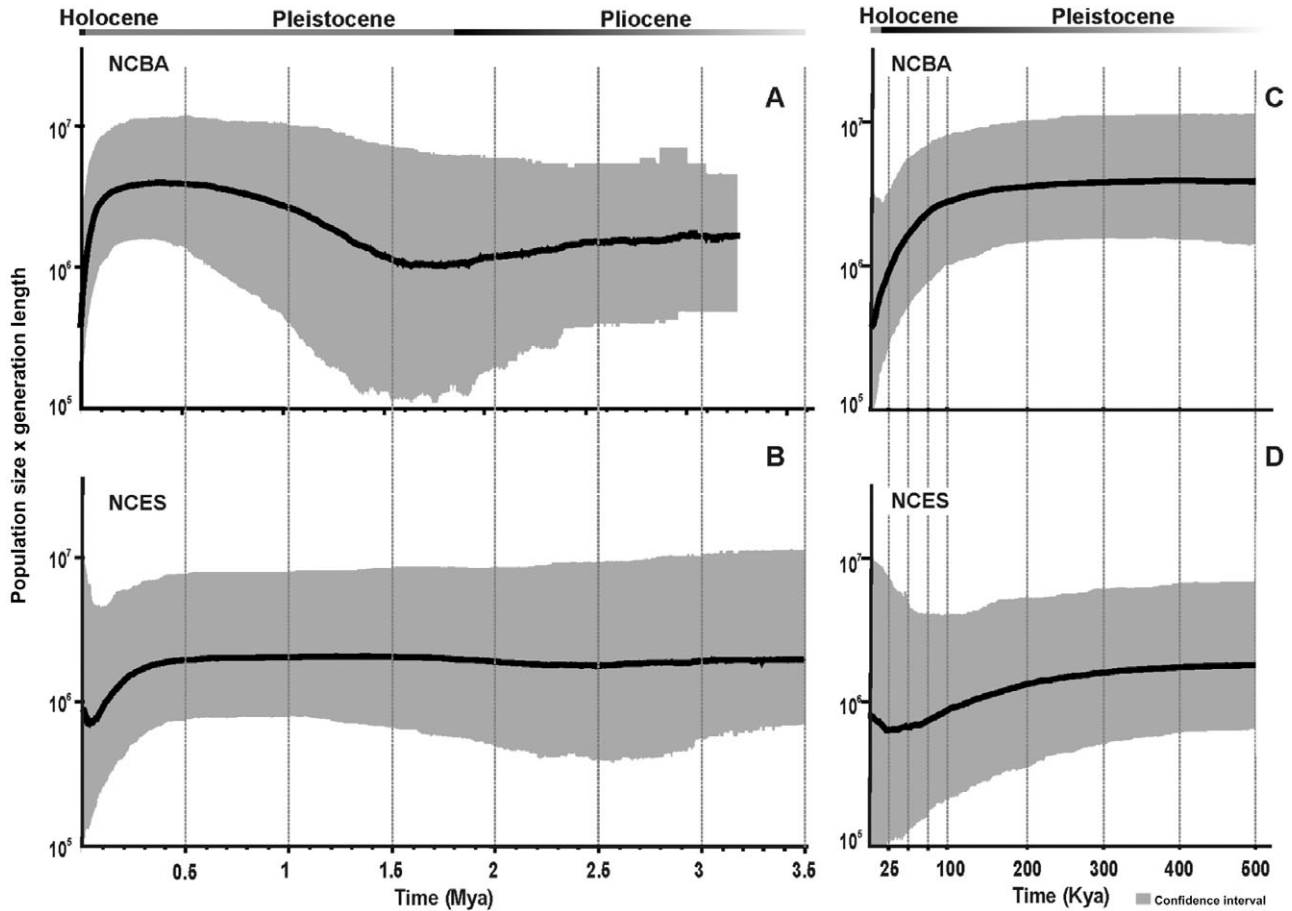
Figure 4. Bayesian Skyline Plot (BSP) for North Clade (NC) groups analysed separately. A, C, BSP for Bahia Clade (NCBA) group (NC3 and NC4 subclade). B, D, BSP for Espírito Santo Clade (NCES) group (NC1 and NC2 subclade). (C) and (D) correspond to an amplified portion of the BSP, highlighting the events since the last 500 000 yBP.

In addition to the founding events which occurred 200 000 yBP, the highly structured pattern of *D. lucida* is probably reinforced by the founding-nest behaviour of apterous reproductive females. Indeed, individuals sampled in geographically close nests share the same haplotype, or very similar haplotypes with low nucleotidic diversity, which was also found within some species of ants with restricted female dispersion (Liautard & Keller, 2001; Doums, Cabrera & Peeters, 2002; Clémencet, Viginier & Doums, 2005).

Given the absence of a winged queen caste in *D. lucida*, we expected that the distribution of mitochondrial haplotypes among the populations would depend on how far the female would walk in order to establish new nests. Doums *et al.* (2002), studying *Diacama cyaneiventre,* another ant without a winged queen caste, found a clear pattern of isolation by distance (IBD), which was also expected within *D. lucida*. However, the partial Mantel test results indicate that historical processes are more important in explaining the genetic structure of *D. lucida*

than the geographical distance. Avise (2009) pointed out that low dispersal species are characterized by strong genealogical structure, but emphasized that 'even in vagile species, historical barriers to dispersal can sometimes be insurmountable', promoting high population structure. Indeed, this pattern was also found in two species of ants with flying queens: *Solenopsis invicta* (Ahrens, Ross & Shoemaker, 2005) and *Formica pratensis* (Goropashnaya *et al*. 2007).

The historic process most probably associated to the present structure of *D. lucida* may be related to the maintenance of isolated populations within refuges formed during the Pleistocene. Schaefer *et al*. (2006) suggest that the forest retraction and consequent formation of fragmented forestal refuges alongside southern Bahia and Espírito Santo States are consequences of the establishment of dry weather in this region, which, in turn, is as a result of the marine regression during the apices of the last glaciation period.

Recent studies (Carnaval & Moritz, 2008; Carnaval *et al.*, 2009) presented evidence for a large central refuge throughout the Late Quaternary, named *Bahia refugium*, which was predicted based on climatic modelling with palynological validation and contrasted with the current distribution of several vertebrate species, such as sloths, lizards, marsupials, sender mice, atlantic rats and frogs. These authors suggest that populations of a given species which remained in stable areas during the Pleistocene (refuges) should present higher DNA variability than those currently found in unstable areas, which should present genetic signature of population expansion, reflecting colonization from adjacent refugial centres. Our results show that, although the populations sampled at the north margin of the Doce River (the centre of the *Bahia refugium*, an stable region) do not present more haplotype and nucleotide diversity than those sampled below the south margin of this river (Table 2), the populations of *D. lucida*, which remained within the centre of the putative *Bahia refugium* (north of the Mucuri River), presented more variability during the Pleistocene (Fig. 4A) than the southernmost populations (Figs 3C and 4B).

Also, the populations below the south margin of the Mucuri River present genetic signatures of population expansion. However, our results point to an expansion of the small populations which remained isolated within putative smaller refuges, in the south area of the *Bahia refugium*, instead of a recent colonization from stable regions of the *Bahia refugium*. Thus, it is possible that the climatic tolerance and realized niche of *D. lucida* differs from those expected for most of the vertebrate species studied so far.

Therefore, our data reinforce the idea that the central region should be considered as a hotspot inside the hotspot of the Atlantic Forest, as proposed by Carnaval *et al.* (2009), once *D. lucida* presents most of the signatures of high diversity during the Pleistocene within stable areas and population growth within the southernmost populations. The difference, however, appears within the evidence that several short populations were maintained during the Pleistocene, accumulating genetic differences over time and resulting into the structured pattern currently shown by *D. lucida*. Thus, we can argue that, for some Atlantic Forest endemic species, especially those resistant to very small fragments of forest, such as *D. lucida*, the small putative refuges were as important as, or even more important than, the central region, for the creation and maintenance of diversity.

IMPLICATIONS TO CONSERVATION

The present area of distribution of *D. lucida* has been suffering a severe regression process, caused mainly by human settlement and destruction of the forests (Campiolo & Delabie, 2008). The loss of habitats promotes the extinction of local populations and, given the restricted female gene flow among populations, a recolonization via natural migration is unlikely.

Our data show that colonies of a same locality usually share the same or highly similar mitochondrial haplotypes, while populations from different fragments present sharply different haplotypes. It is crucial to note that gene flow of the winged male should reduce the effects of isolation, but the loss of mtDNA diversity by local extinction will never be replaced.

As with *D. lucida*, other species populations may have remained isolated within very small refuges during the Pleistocene, generating a lot of variability and probably leading to a rise in the species. Given this assumption, the destruction of habitats which currently maintain *D. lucida* populations could lead to the extinction of highly differentiated populations of unstudied species, or even of unknown species with strong local endemism.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Figure S1. Rooted phylogenetic tree.
Figure S2. Median-joining haplotype network.
Table S1. PCR primers.
Table S2. Details of the Bayesian Skyline Plot analyses.
Table S3. GenBank accession reference.

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