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Research Article

Genetic diversity and structure of *Atta robusta* (Hymenoptera, Formicidae, Attini), an endangered species endemic to the *restinga* ecoregion

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Abstract

The genetic diversity and structure of the ant *Atta robusta* were assessed by ISSR (inter-simple sequence repeats) in 72 colonies collected from 10 localities in the Brazilian states of Espírito Santo (48 colonies) and Rio de Janeiro (24 colonies). The ISSR pattern included 67 bands, 51 of them (76.1%) polymorphic. Analysis of molecular variance (AMOVA) revealed a high level (57.4%) of inter-population variation, which suggested a high degree of genetic structure that was confirmed by UPGMA (unweighted pair-group method using an arithmetic average) cluster analysis. The significant correlation between genetic and geographic distances ($r = 0.64$, $p < 0.05$) indicated isolation that reflected the distance between locations. Overall, the populations were found to be genetically divergent. This finding indicates the need for management plans to preserve and reduce the risk of extinction of *A. robusta*.

Keywords: endemism, endangered, ISSR, population genetics, *restinga*.

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Introduction

The ant *Atta robusta* (Borgmeier, 1939) maintains a mutualistic symbiotic relationship with a fungus on which it feeds (Fowler, 1995; Currie, 2001). This species was first described as *Atta sexdens robusta* by Borgmeier in 1939 and later elevated to species level by Gonçalves (1942). *Atta robusta* has a very restricted geographic distribution, occurring only in southeastern Brazil, more precisely, in *restingas* in the states of Espírito Santo and Rio de Janeiro (Gonçalves, 1960; Teixeira *et al.*, 2003, 2004), but not in forest ecosystems near *restinga* vegetation (Teixeira *et al.*, 2003). This species is included in the Red List of Brazilian Fauna Threatened with Extinction (Machado *et al.*, 2005) because of its endemism and dependency on vegetation (Teixeira and Schoereder, 2003) and the fragmentation of *restinga* environments (Rocha *et al.*, 2007; Simon *et al.*, 2012).

The term *restinga* represents a set of physiognomically distinct plant communities under marine influence (Sugiyama, 1998). *Restingas* are geologically recent (Quaternary) ecosystems and species that colonize them are mainly from other ecosystems such as the Atlantic Forest and Caatinga, but with phenotypic variations that differ from those expressed in their original environments (Freire, 1990). *Restingas* cover large areas of the coasts of the Brazilian states of Rio Grande do Sul, Rio de Janeiro and Espírito Santo (Henriques *et al.*, 1987) and have sandy soils characterized by leaching, high decomposition rates, aluminium saturation and low availability of nutrients (Lacerda *et al.*, 1993). In this biome, nests of *A. robusta* are found only where there is arboreal plant cover, possibly because of the particular needs of this species, such as adequate temperature and moisture for nest development (Teixeira and Schoereder, 2003).

Despite their ecological importance, *restingas* are extensively occupied and exploited by humans (Galindo-Leal and Câmara, 2003; Rocha *et al.*, 2003, 2005). Much of the area of this biome is lost annually as a result of urbanization (Rocha *et al.*, 2007), logging, burning (Teixeira *et al.*, 2005), tourism, cultivation and grazing. On the other hand, this biome is particularly difficult to restore because of its soil characteristics (Rocha *et al.*, 2005) and constant human encroachment. Currently, only a small portion of *restinga* land is protected (Simon *et al.*, 2012).

Although easily confounded with other pest species, *A. robusta* does not invade cultivated areas (Teixeira *et al.*, 2003). However, the quantity and variety of plant material consumed by this species mean that *A. robusta* is important in the maintenance of *restinga* communities that are part of the larger Atlantic Forest biome. In this biome, *A. robusta* plays an important role in seed dispersal and germination, in the spatial dynamics of seedlings and in soil modification (Teixeira, 2007).

Since genetic data can contribute to species conservation and considering the importance of *A. robusta* in *restinga* biology, the goal of this study was to estimate the

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genetic variability and the degree of structuring in colonies of *A. robusta*.

Material and Methods

Biological material

Workers of *A. robusta* were collected at six locations in the state of Espírito Santo (48 colonies) and four in the state of Rio de Janeiro (24 colonies); all of these locations were in *restingas* (Figure 1 and Table 1). The ants were fixed in absolute ethanol immediately after collection. In the laboratory, the absolute alcohol was changed and the samples were stored at -80 °C until DNA extraction.

DNA extraction and amplification

The heads and mesothoraces of *A. robusta* workers were used for total DNA extraction (one extraction per colony) according to the protocol described by Waldschmidt *et al.* (1997). Quantification and verification of the integrity of the extracted DNA were done by gel electrophoresis in 0.8% agarose. The DNA obtained was diluted to a concentration of 10 ng/μL and used in amplification reactions with ISSR (inter-simple sequence repeat) primers (UBC set). Initially, 99 ISSR primers were tested using DNA from two individuals. Of these, ten (UBC 807, 809, 823, 825, 834, 840, 845, 855, 857 and 889) were selected to amplify DNA from all samples because they yielded high resolution

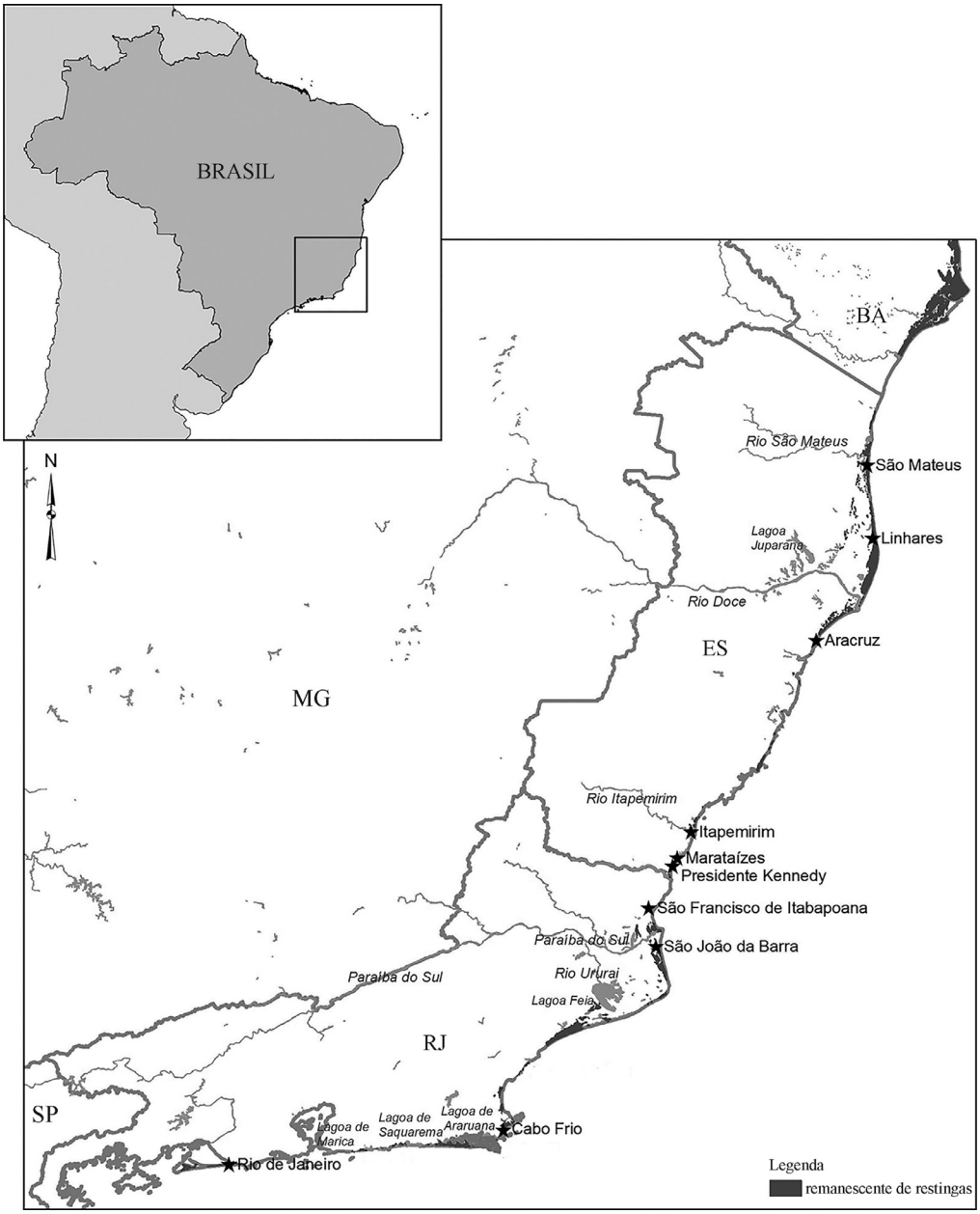

Figure 1 - Geographical locations (*) of the municipalities in the states of Espírito Santo (ES) and Rio de Janeiro (RJ) where *A. robusta* was collected.

Table 1 - Locations sampled and the number of *A. robusta* colonies analyzed in this study.

State	Location (Code)	No. of colonies
Espírito Santo	Aracruz (AR)	9
	Itapemirim (IT)	9
	Linhares (LI)	9
	Marataízes (MR)	3
	Presidente Kennedy (PK)	8
	São Mateus (SM)	10
	Subtotal	48
Rio de Janeiro	Cabo Frio (CF)	3
	Rio de Janeiro (RJ)	11
	São Francisco de Itabapoana (SF)	5
	São João da Barra (SJ)	5
	Subtotal	24
	Total	72

bands and ISSR patterns that were easily distinguishable between the two individuals evaluated.

The polymerase chain reaction (PCR) amplification protocol consisted of an initial denaturation step at 94 °C for 3 min, followed by 39 cycles of 1 min at 92 °C, 2 min at the primer annealing temperature (Table 2) and 2 min at 72 °C, followed by a final extension at 72 °C for 7 min. Amplifications were done in volumes of 25 μL that contained 0.2 μM of primer, 8 μM dNTP, 0.5 U of *Taq* DNA polymerase (Promega, Madison, WI, USA), 1X PCR buffer and approximately 20 ng of genomic DNA. All reactions included a negative control that contained all components of the PCR except for template DNA. To ensure accurate scoring and reproducibility, the amplifications were repeated twice and the data were analyzed independently by two persons.

The PCR products and the 1 kb ladder (Invitrogen, Carlsbad, CA, USA) were separated by electrophoresis on 1.5% agarose gels (w/v) with 0.2 μg/mL ethidium bromide immersed in 1X TBE (Tris-borate EDTA). The gels were photographed after staining.

Statistical analysis

The amplification products were coded in a binary matrix by assigning 1 for presence and 0 for absence of bands. The program Popgene version 1.32 (Yeh *et al.*, 1999) was used to determine the percentage of polymorphic loci. A matrix with the complement of the Jaccard dissimilarity coefficient (Jaccard, 1908) was obtained and subjected to analysis of molecular variance (AMOVA; Excoffier *et al.*, 1992), to assess intra- and inter-population diversity in two hierarchical levels, and to estimate the pairwise genetic distance between locations. The genetic distance matrix was also examined by cluster analysis using the UPGMA (unweighted pair group method using an arithmetic average) algorithm. The Mantel test was used to examine the possible correlation between geographic and genetic distances for the different locations. These analyses were done using the GENES program (Cruz, 2013).

Results

Amplification using the 10 selected ISSR primers resulted in 67 bands (an average of 6.7 bands/primer). The greatest number of bands (9) was obtained with the primers UBC 809, 840 and 857 whereas the lowest number (4) was observed for the primers UBC 807, 825 and 845. The average polymorphism among the samples was 76.1% (51 polymorphic bands) (Table 2).

The genetic distance between samples ranged from 0.04 (Marataízes and São João da Barra) to 0.78 (Aracruz and Cabo Frio) (Table 3). Two groups were identified in the cluster analysis (Figure 2): samples from Aracruz, São Mateus and Linhares, all of which were from Espírito Santo and geographically close to one another, formed the first group, while the remaining samples from Espírito Santo and Rio de Janeiro formed the second group.

The Mantel test identified a significant correlation between genetic and geographic distances ($r = 0.64$, $p < 0.05$), and AMOVA showed that most of the genetic variation (57.4%) was attributable to differences among populations (Table 4).

Discussion

Cluster analysis yielded two well-resolved groups of *A. robusta*, one composed only of samples from three geographically close localities in the *restinga* of Espírito Santo state, and the other containing all of the remaining samples. The Mantel test confirmed this analysis and showed a significant correlation between genetic and geographic distances. These results suggested that *A. robusta* experiences isolation by distance: the greater the geographic distance

Table 2 - Data on the primers used to amplify total DNA of *A. robusta*. T = annealing temperature.

Primers	T (°C)	Number of bands	Number of polymorphic bands	% polymorphism
UBC 807	45.0	4	2	50
UBC 809	55.2	9	5	55
UBC 823	55.2	6	2	33
UBC 825	53.3	4	3	75
UBC 834	51.2	7	7	100
UBC 840	45.2	9	6	66
UBC 845	53.3	4	4	100
UBC 855	55.2	7	7	100
UBC 857	58.5	9	8	88
UBC 889	53.3	8	7	87

Table 3 - Matrix of pairwise genetic distances between the locations where samples of *A. robusta* were collected.

	AR	SM	IT	LI	MR	PK	SF	SJ	RJ	CF
AR	**									
SM	0.09	**								
IT	0.69	0.66	**							
LI	0.10	0.12	0.63	**						
MR	0.76	0.71	0.25	0.67	**					
PK	0.74	0.71	0.12	0.68	0.10	**				
SF	0.77	0.72	0.08	0.70	0.26	0.06	**			
SJ	0.69	0.66	0.13	0.63	0.04	0.10	0.19	**		
RJ	0.76	0.73	0.27	0.71	0.28	0.24	0.34	0.09	**	
CF	0.78	0.72	0.13	0.68	0.30	0.12	0.10	0.18	0.39	**

AR: Aracruz; SM: São Mateus; IT: Itapemirim; LI: Linhares; MR: Marataízes; PK: Presidente Kennedy; SF: São Francisco de Itabapoana; SJ: São João da Barra; RJ: Rio de Janeiro; CF: Cabo Frio.

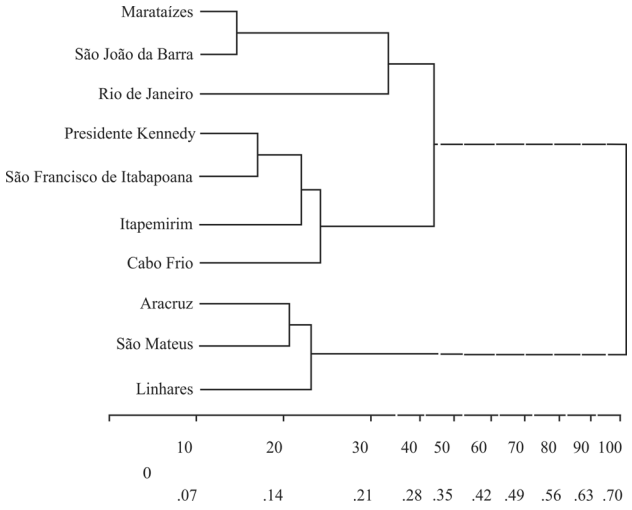

Figure 2 - Dendrogram generated by UPGMA based on the matrix of genetic distances between the locations where samples of *A. robusta* were collected.

Table 4 - Analysis of molecular variance (AMOVA) among the locations where samples of *A. robusta* were collected.

Source of variation	d.f.	Sum of squares	Variance components	Variation (%)
Among populations	9	5.785	0.082	57.36
Within populations	62	3.786	0.061	42.64
Total	71	9.571	0.143	100.00

between any two locations, the greater the genetic distance between them as well.

The genetic structure of *A. robusta* was also confirmed by AMOVA, which showed that most of the genetic variability (57.4%) was detected among the populations. This high genetic structure may reflect restricted or absent gene flow between localities as a result of fragmentation of the *restinga* biome. Indeed, the formation of these two groups can be explained by the existence of an arid region between southern Espírito Santo and northern Rio de Janeiro (Jackson, 1978) that would prevent the establishment of colonies of *A. robusta* in this region, thereby limiting the gene flow between populations. The fact that no samples of *A. robusta* were found in this region (despite sampling efforts in the field), together with the importance of the climatic conditions of this region in affecting the distribution and differentiation of lizards of the genus *Enyalius* (Jackson, 1978), support the hypothesis that the emergence of this drier region led to the isolation of populations and their subsequent genetic differentiation.

According to Jackson (1978), the more arid climate in this region (caused by the weak rain shadow on the coastal side of the mountains) and the penetrance of the Itapemirim River through southern Espírito Santo could have favored an invasion by the cerrado biome in extreme southern Espírito Santo. Thus, a dry period may have isolated part of the original population of *A. robusta* by interposing a drier region with more open vegetation in which *A. robusta* could not survive. In addition, the regions between northern Espírito Santo and southern Bahia (among others), as well as those located in the Serra dos Órgãos (Rio de Janeiro), may have functioned as large-scale refugia during the Pleistocene (Jackson, 1978), thereby favoring the establishment of *A. robusta*.

According to Teixeira *et al.* (2003), *A. robusta* is restricted to the *restinga* vegetation of the Brazilian coast extending from the southeastern state of Rio de Janeiro up to the Reconcavo Baiano, more formally known as the Moist Oriental Tertiary Brazilian Coast, in the state of Bahia (Schobbenhaus, 1984). These authors also suggested that *A. robusta* does not disperse southward because of the Serra do Mar mountain range present in the southern part of Rio de Janeiro state. Indeed, this species has not been found in *restingas* of the states of São Paulo (Mariconi, 1965), Santa Catarina (Bonnet and Lopes, 1993) and Paraná (Lopes, 1998). The superficial water table in São Paulo state could

also contribute to the absence of *A. robusta* along the coast of this state since this water may interfere in nest development (Mariconi, 1965). Teixeira *et al.* (2003) emphasized that there is no record of *A. robusta* in the state of Bahia, despite the lack of any evident geographical barrier to impede the northward dispersion of this species beyond Espírito Santo.

The limited range of *A. robusta* suggests that this species may be adapted to specific environmental conditions. This, together with the fact that no other *Atta* species occur in this biome, indicates the need for further studies to explain the evolutionary strategies that differentiate *A. robusta* from the other species of this genus, especially with regard to the colonization of *restingas* (Teixeira *et al.*, 2003). Endemism in *restingas* is probably associated with Pleistocene climatic oscillations and changes in sea level (Jackson, 1978), and several other taxa are also endemic to this biome. For example, Carvalho-e-Silva *et al.* (2000) identified five amphibian species (*Leptodactylus marambaiae*, *Xenohyla truncata*, *Scinax littorea*, *Bufo pygmaeus* and *Scinax agilis*) endemic to *restingas* of the Brazilian southeastern and south-northeastern regions. Additionally, Rocha *et al.* (2005) identified one species of reptile (*Liolaemus lutzae*), three species of birds (*Formicivora littoralis*, *Mimus gilvus*, *Schistochlamys melanopis*) and one species of rodent (*Trinomys eliasi*), all considered endangered, in the *restingas* of the Serra do Mar and Central da Mata Atlântica biodiversity corridors.

Atta robusta inhabits a very restricted environment that is being rapidly occupied by humans. This reduces the amount of available vegetation that in turn affects the distribution of *A. robusta* nests. Indeed, Teixeira and Schoereder (2003) found that arboreal plant cover has a positive effect on the density of *A. robusta* nests, whereas fragmented environments hinder the nesting ability of this species. These authors suggested that the dependency of *A. robusta* on areas with high vegetation cover could be a response to the species' particular needs in a *restinga* environment. Accordingly, Teixeira *et al.* (2005) found no *A. robusta* nests in environments degraded by burning. Once a *restinga* has been altered by human activity it is very difficult for it to be restored (Rocha *et al.*, 2005). Thus, the fragmentation of *restingas* may have contributed to the genetic differentiation estimated for *A. robusta* in the present study.

Using the same samples analyzed in the present study, Simon *et al.* (2012) examined the nest density in different fragments of *restinga* as well as the environmental conditions necessary for colonization by *A. robusta*. These authors detected a greater abundance of nests in larger, more preserved fragments. They also reported that the areas sampled in Aracruz, Itapemirim and Marataízes (Espírito Santo) and São de Itaboapoana and São João da Barra (Rio de Janeiro) were in a critical condition with regard to the conservation of *A. robusta*: 38.8% of the nests sampled were in good condition, 18.8% were partially preserved and 36.3% were in a critical condition.

In addition to characteristics such as a tendency to salinity, high sand content and low levels of water and nutrient retention, the temperature in open *restinga* can reach more than 30 °C at a depth of 30 cm (Franco *et al.*, 1984). These characteristics may affect the establishment and initial development of *A. robusta* nests in this biome since the nests of leafcutter ants have an initial depth of only 8.5-18 cm. More generally, these characteristics mean that species that live in *restingas*, particularly *A. robusta*, recover slowly from disturbances caused by humans, and this could contribute to the decline of this species. Another important consideration is that the moisture inside nests may be more uniform in *restingas* that offer greater vegetation cover (Franco *et al.*, 1984). This improved moisture could result in more resources for *A. robusta* (Teixeira and Schoereder, 2003) and contribute to the preservation of this species.

The results of this study provide additional information on the genetic organization and divergence of *A. robusta* populations. This knowledge may be useful in future management plans for this endemic species in the *restinga*s of Rio de Janeiro and Espírito Santo. The preservation of *A. robusta* must attempt to maintain the highest possible number of colonies in their original habitat, *i.e.*, protect the two genetic subgroups identified in this study. Since *A. robusta* nests only in areas covered with arboreal plants, recovering the vegetation in fragmented areas in order to construct corridors between larger areas could contribute to this species preservation. Finally, since remnant populations of *A. robusta* can adapt to the conditions of the *restinga* ecoregion, preserving whole *restinga* environments is also very important.

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