POPULATION GENETICS OF *Atta sexdens rubropilosa* (HYMENOPTERA: FORMICIDAE)

Genética de poplaciones de *Atta sexdens rubropilosa* (Hymenoptera: Formicidae)

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ABSTRACT

The genetic variability of *Atta sexdens rubropilosa* leaf-cutting ants collected from five brazilian localities was evaluated with PCR-RAPD technique. We used 15 primers producing 148 fragments of which 123 (83.11 %) contained polymorphisms. The estimated Shannon index was 0.3836 ± 0.2335 showing that these ants possess high genetic diversity. The G_{ST} value was 0.2372 and Φ_{PT} = 0.184, indicating that the analyzed populations are moderately differentiated and 82 % of the variation obtained occur within populations. Although Mantel's test had shown correlation between genetic distances and geographic was observed that Ivatuba and Itambé (33.8 km) have the small geographical distance and the largest genetic distance. The lower genetic distance was estimated for Maringá and Ivatuba but this localities have a small geographic distance (42.3 km), indicating that there are no barriers for mating among reproducers in these populations. The high degree of polymorphism (83.11 %) and the ability to cross among the populations in the studied regions indicate that this species of leaf-cutting ant is well adapted to the region; therefore, integrated control programs can be developed.

Keywords: genetic variability, leaf-cutting, PCR-RAPD.

RESUMEN

La variabilidad genética de las hormigas *Atta sexdens rubropilosa* colectadas en cinco lugares distintos de Brasil fueron evaluados por la técnica PCR-RAPD. Un total de 15 primers produjeron 148 fragmentos, de los cuales 123 fueron polimórficos, lo que corresponden al 83,11 %. La estimación de la diversidad genética por el índice de Shannon fue 0,3836 y el desviación estándar fue de ± 0,2335. Estos valores demuestran una alta diversidad

genética. El valor de G_{ST} fue 0,2372 y Φ_{PT} = 0,184 lo que indica que las poblaciones están moderadamente diferenciadas y que el 82 % de la variación obtenida se produce dentro de las poblaciones. Aunque la prueba de Mantel ha demostrado una correlación entre la distancia genética y geográfica se observó que Ivatuba e Itambé (33,8 km) tiene una pequeña distancia geográfica y la mayor distancia genética. La distancia inferior genética fue estimada para Maringá e Ivatuba pero estas localidades cuentan con una distancia geográfica pequeña (42,3 km), lo que indica que no hay barreras para el apareamiento entre los reproductores en estas poblaciones. El alto valor de polimorfismo (83,11 %) y la capacidad de emparejamiento entre las poblaciones presentes en las regiones estudiadas, indican que esta especie de hormiga cortadora está bien adaptada a la región, y deben ser desarrollados programas integrados de control si se convierten en plagas.

Palabras clave: hormigas cortadoras, PCR-RAPD, variabilidad genética.

INTRODUCTION

Leaf-cutting ants are considered important pests in the neotropics because they have a wide geographical distribution and are responsible for cutting a wide variety of native and cultivated plants. In particular, *Atta sexdens rubropilosa* species (Forel, 1908) harm *Eucalyptus* and *Pinus* spp. (Boaretto *et al.*, 1997) by cutting leaves and tender branches and are capable of completely destroying plants (Oliveira, 1996).

This occurs mainly in homogeneous stands, which favor the development of pests, such as ants of the *Atta* and *Acromyrmex genera* (Della Lucia, 1993).

Forestry companies have used chemicals to control leaf-cutting ants. However, economic and environmental factors have led companies to improve the operational efficiency of chemical control techniques. In recent years, there has been an increase in the number of jobs related to biological and cultural control, especially in relation to plant resistance, in an attempt to identify alternatives to chemical control strategies or formulate combinations of different control strategies (Della Lucia, 1993).

Ants also have a variety of ecological functions as seed dispersers, contributing to the reforestation of many ecosystems and promoting their germination, removed the fruit pulp (Peternelli *et al.*, 2004). They are responsible for pruning some plants, promoting their vegetative growth and play an important role in soil aeration (Hölldobler and Wilson, 1990), and incorporate organic matter on earth, making it fertile (Moutinho *et al.*, 2003).

In general, ants provide the unique opportunity to investigate various biological, evolutionary, and population issues because they form highly complex colonies due to their diverse social classes. Ants may be used to conduct ecological and population genetic structure studies, and they also provide rich material for investigating kin selection and altruism (Brian, 1983; Sudd and Franks, 1987; Hölldobler and Wilson, 1990). Ants are haplodiploid organisms, as the males emerge from parthenogenesis and females from fertilized eggs. The occurrence of two ploidy levels can be used as an important tool for genetic analysis (Diehl *et al.*, 2002).

Atta colonies usually consists a queen (mongyne) and thousands or millions of sterile workers of different sizes and shapes (Hölldobler and Wilson, 2009). The adults, with rare exceptions, are females and are divided into at least two castes: fertile females or

queens, whose primary function is to egg laying and sterile females, which perform all the other colony activities, such as water collection and food, feeding the young and queen, construction and defense of the nest (Wilson, 1976). Males constitute an additional caste and usually appear only once a year, in mating season, which occurs through the completion of the nuptial flight (Hölldobler and Wilson, 2009). The queen, after three to eight fertilized by males during the mating flight, falls to the ground and removes their wings to begin building the new nest and male die after mating (Forti, 1985).

Some authors have argued that diploid organisms have greater genetic variability than haplodiploid organisms (Pamilo *et al.*, 1978; Falcão and Contel, 1990). Graur (1985), concluded that the level of sociality is more important than haplodiploidy in determining genetic diversity in hymenoptera.

Since the 1960s, isoenzyme analysis has been the dominant method used for the evaluation of genetic variation in natural populations. Other techniques are currently available for the study of genetic diversity, such as random amplification of polymorphic DNA analysis (RAPD), but all techniques have advantages and disadvantages (Ferreira and Grattapaglia, 1998). RAPD is based on the identification of polymorphic DNA randomly amplified using a single primer with an arbitrary sequence, which is capable of amplifying DNA sequences contained between two annealing sites (Welsh and Mcclelland, 1990). Although this technique uses random sequences, many studies have shown that it can be used to effectively identify insect populations (Lopes-da-Silva *et al.*, 2004) and for genetic variability studies.

Using isozyme polymorphisms (Cantagalli *et al.*, 2010) analyzed the population structures of *Atta capiguara* (honeybee-brown ants) in the Tapejara region (PR) and found low genetic variability for this species. Thus, the RAPD technique was used in this study to analyze the genetic variability and population structure of the *A. s. rubropilosa* leaf-cutter ant to increase the understanding of ant population genetics and improve the management of this species.

MATERIALS AND METHODS

Biological sample collection

Atta sexdens rubropilosa soldiers, which are easily identified by their characteristic lemon grass smell due to a gland with an alarm function in the head, were collected from five different regions in the states of Paraná and São Paulo, Brazil. In northwestern Paraná, samples were collected from Maringá (23°26' S, 51°56' W), Itambé (23°39'39" S, 51°59'24" W), and Ivatuba (23°37'08" S, 52°13'15 W). In São Paulo, the samples were collected from Presidente Prudente (22°07'04" S and 51°22'57" W) and Dracena (21°28'57" S, 51°31'58" W). Collection localities can be visualized in Fig. 1.

In Maringá, Itambé, and Ivatuba, four nests were sampled at each location, and in Presidente Prudente and Dracena, two nests were sampled per site, for a total of 16 nests. The collected material was stored in plastic bottles at -20 °C.

DNA isolation

Total DNA was extracted from five individuals from each nest, with the exception of one nest from Ivatuba, where it was only possible to extract DNA from four individuals,

Figure 1. Map of South America, Brazil, States of Paraná (PR) and São Paulo (SP) indicating locations of *A. s. rubropilosa* collection.

for a total of 79 samples. Only the heads of soldiers were used to prevent contamination with nucleases that are present in the digestive tract of these insects.

The method used for total DNA extraction was a modified version of that described by (Yu *et al.*, 1993). The heads were homogenized in 1,5 ml microcentrifuge tubes containing 300 µl of extraction buffer [200 mM Tris-HCl (pH 8,0), 0,5 % SDS, 250 mM NaCl, 50 mM EDTA, and 100 mg/ml Proteinase K]. After 30 min of incubation at 65 °C, the material was centrifuged for 10 min (10,000 x g) and the supernatant was transferred to a new tube. An equal volume of chloroform: isoamyl alcohol (24:1) was then added. After homogenization and centrifugation for phase separation, the upper phase was transferred to a new tube. The DNA was precipitated with 250 µl of cold isopropanol and incubated at 20 °C overnight. The precipitated DNA was separated by centrifugation at 10,300 x g for 10 min. After discarding the supernatant, the precipitate was washed with 1.0 ml of cold 70 % ethanol and left to dry at room temperature. After drying, the DNA was resuspended for two hours in 50 µl of TE buffer, treated with RNAse (10 mg/ml), and incubated at room temperature for more than 2 hours before storage at -20 °C.

The DNA quality was evaluated and quantified on 0.8 % agarose gels with 1X TAE buffer. The amount of DNA present in each sample was estimated by comparison with known concentrations of a graded DNA standard (λ phage). The gels were stained in ethidium bromide bath (0.5 mg/mL), and the gel images were visualized under ultraviolet light and captured with the EDAS system (Kodak 1D Image Analysis 3.5, New York, USA).

Amplification of fragments via PCR-RAPD, separation, and visualisation of products

To evaluate the genomes of the *Atta sexdens rubropilosa* populations, 15 primers were produced by Operon Technologies Inc., Alameda, CA, USA (OPA-01, OPA-02, OPA-03, OPA-04, OPA-07, OPA-10, OPA-11, OPA-14, OPA-18, OPA-19, OPB-11, OPB-12, OPB-17, OPC-20, and OPM-3).

The amplification conditions were based on the methodology described by (Williams *et al.*, 1990). For a reaction volume of 20 µl, it was used 1X Tris-KCl [20 mM Tris-HCl (pH 8.4) and 50 mM KCl], 2.5 mM $MgCl_2$, 0.3 mM of primer, 0.1 mM each dNTP, one

unit of Taq DNA polymerase (Invitrogen), and 20 ng of template DNA. Reactions were performed in an Eppendorf Mastercycler® Gradient thermocycler, as follow 96 °C for five minutes followed by 45 cycles of 30 seconds at 94 °C, 45 seconds at 35 °C, and one minute at 72 °C, with a final extension at 72 °C for seven minutes. A negative control reaction (with no DNA template) was used.
The amplified products were separated in a 1.7 % agarose and stained in an ethidium bromide bath. The DNA fragments were visualized under an ultraviolet light, and the images were captured with the EDAS system (Kodak 1D Image Analysis 3.5).

Population genetics analysis

The data analysis involved comparison of the molecular weights of the fragments (bands) obtained from the genomic DNA. Individual bands with identical molecular weights (located on the same loci) were compared within and between populations and designated as present (1) or absent (0) for each individual analyzed.
Genetic variability within and between populations was determined by the percentage of polymorphic loci. The genetic diversity of the populations studied was estimated with the Shannon index (I) (Lewontin, 1972), G_{ST} values were also calculated. Nei's (1978) genetic distance had been used to UPGMA cluster analysis (unweighted pair-group method using an arithmetic average) and construct a dendrogram. For these analyses, the Popgene 1.31 program was used (Yeh *et al.*, 1999).
GenALEX 6.5 (Peakall and Smouse, 2012) software was used to estimate AMOVA (analysis of molecular variance) and phi'PT (Φ_{PT}); to construct genetic relationships tree among *A. s. rubropilosa* populations based on Nei and Li/Dice; to estimate percentage confidence level in the bootstrap analysis (1,000 replicates); spatial analyses involving binary data were performed estimating principal coordinate analysis (PCA) and Mantel test (1967).

RESULTS

The amount of extracted DNA was ranged between 10 and 40 ng. To verify the genetic variability of *Atta sexdens rubropilosa* species collected from different region and the ability to amplify the extracted DNA via PCR-RAPD, 80 primers were initially tested, of which 15 were used. Table 1 shows the nucleotide sequences of the selected primers, the numbers of the total and polymorphic fragments, and the sizes of the amplified fragments.

Primer	Nucleotide sequence	Fragments number	Polymorphic fragments number
OPA- 01	5'- CAGGCCCTTC -3'	09	09
OPA -02	5'- GTGACGTAGG -3'	09	07
OPA- 03	5'- AGTCAGCCAC -3'	13	11
OPA- 04	5'- AATCGGGCTG -3'	08	06
OPA- 07	5'- GAAACGGGTG -3'	17	15
OPA- 10	5'- GTGATCGCAG -3'	04	03

Table 1. Primers, nucleotide sequences, numbers of fragments per primer, and numbers of polymorphic fragments amplified via RAPD-PCR from DNA extracted from *Atta sexdens rubropilosa* populations.

The number of clear and reproducible fragments generated by the primers in all populations studied ranged from four to 17 fragments, with an average of 9.8 fragments per primer, and the sizes of the amplified products were between 200 and 5000 bp (Fig. 2). The 15 primers used in the RAPD-PCR reactions produced 148 fragments. It was observed that of out the total number of fragments analyzed, 123 (83.11 %) displayed polymorphisms.

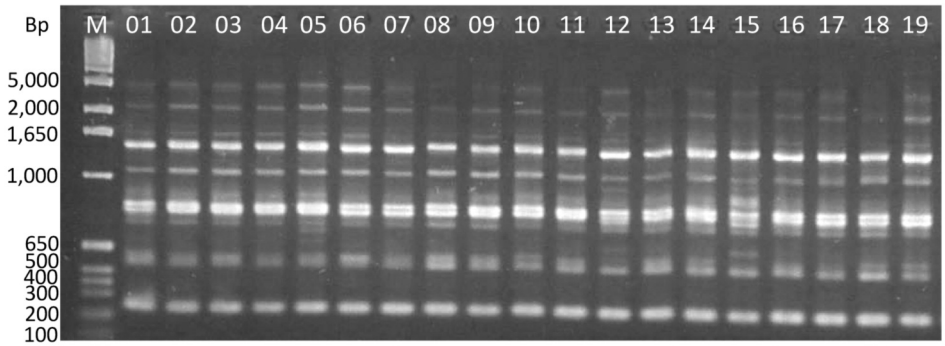

Figure 2. RAPD electrophoretic profiles of *Atta sexdens rubropilosa*. Fragments were amplified with the OPA-03 primer with samples from Maringá (01 to 19). M = molecular weight DNA marker (DNA Ladder, Invitrogen).

The estimated genetic diversity obtained with the Shannon index (0.3836) and the standard deviation (± 0.2335) indicated that these populations had high genetic diversity. The high value of G_{ST} (0.2372) and Φ_{PT} = 0.184 (significant probability at 0.010) showed that *A. s. rubropilosa* populations are moderately differentiated. G_{ST} can be considered an estimate of F_{ST} when it is assumed subpopulations are in Hardy-Weinberg equilibrium (F_{IS} = 0). Therefore, considering Wright (1984) can be considered that G_{ST} values between 0.15 and 0.25 show that populations are moderately differentiated. AMOVA analysis showed that 82 % of the variation obtained with RAPD markers occurs within populations and 18 % among populations.

Principal Component Analysis (PCA) estimated by the covariance matrix showed that 71.84 % of the variation is explained by the coordinates 1 and 2 (Fig. 3). The first three coordinates explained 81.30 % of the variation. The coordinates 1, 2 and 3 correspond to 58.51 %, 13.33 % and 9.46 % respectively of variation.

Nei's genetic distance values among the five populations analyzed indicated that the populations from Presidente Prudente and Ivatuba had highest genetic distance (Table 2) but not geographical (215 km, Table 3), whereas the populations from Maringá and Ivatuba had the lowest genetic distance (Table 2) but not geographical (46.5, Table 3). The correlation between genetic distance and geographical distance was evaluated by Mantel's test. Fig. 4 shows that there is correlation between these variables (R = 0.344, p = 0.001). A dendrogram based on the arithmetic complement of Nei's genetic distance (1978) (Fig. 5) shows that *A. s. rubropilosa* analyzed can be separated into two main groups. The first is formed by Ivatuba-PR, Maringá-PR and Presidente Prudente-SP, the second has populations from Dracena-SP and Itambé-PR (Fig. 5).

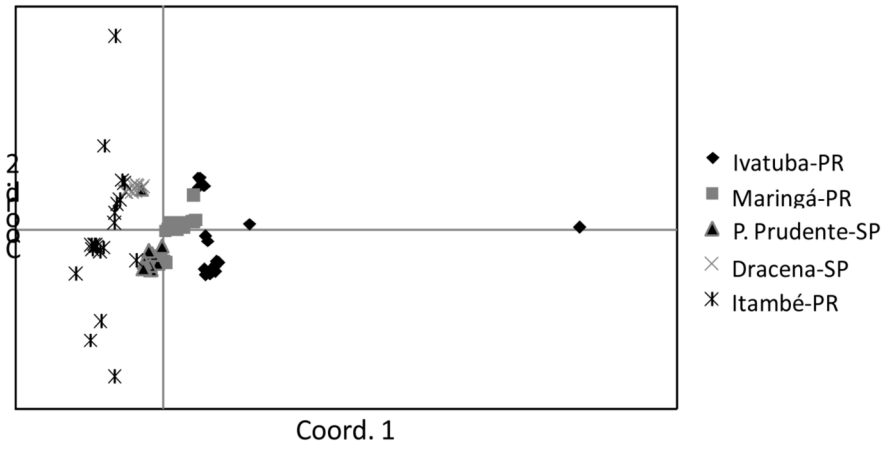

Figure 3. Principal component analysis (PCA) shows the coordinates 1 and 2 corresponding to 71.84 % of the variability.

Pop/ID	Ivatuba	Maringá	P. Prudente	Dracena	Itambé
Ivatuba	***	0.9583	0.8632	0.9346	0.8966
Maringá	0.0426	***	0.9094	0.9546	0.9264
P. Prudente	0.1472	0.0950	***	0.9011	0.9299
Dracena	0.0676	0.0464	0.1042	***	0.9068
Itambé	0.1092	0.0764	0.0727	0.0979	***

Table 2. Values of genetic identity and Nei's genetic distance (1978) obtained with the Popgene 1.31 program for the five *Atta sexdens rubropilosa* populations. *The genetic identity values are presented above the diagonal and Nei's genetic distance values are below the diagonal.

Km	Ivatuba (PR)	Maringá (PR)	P. Prudente (SP)	Dracena (SP)	Itambé (PR)
Ivatuba	***	46.5	215	316	36.5
Maringá		***	173	273	42.1
P. Prudente			***	102	212
Dracena				***	312
Itambé					***

Table 3. Geographic distance (Km) between sampling sites of *Atta sexdens rubropilosa*. SP = São Paulo state; PR = Paraná state.

UPGMA clustering based on the arithmetic complement of the Jaccard coefficient was not used because the values were not significant at the 70 % level by bootstrap analysis with 1,000 repetitions.

DISCUSSION

Carvalho and Vieira (2001) reported that the amount of DNA in relation to the primer is one of the key factors for successful PCR because amplification may not occur if the

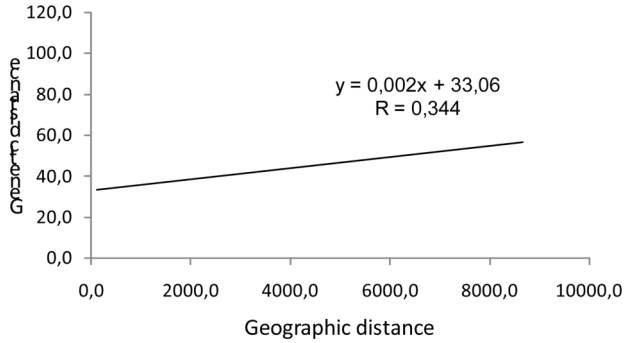

Figure 4. Mantel test showing that there is correlation (p = 0,001) between genetic distance and geographic distance for *A. s. rubropilosa* populations analyzed (999 permutations).

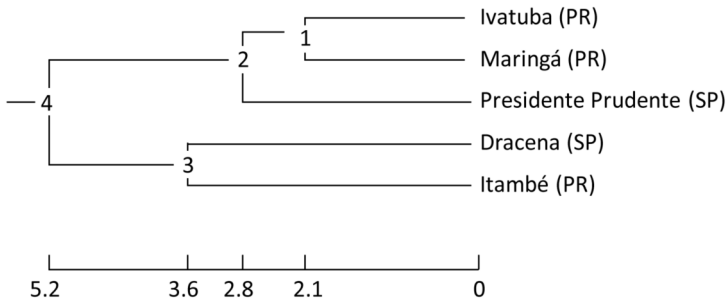

Figure 5. Dendrogram based on the arithmetic complement of Nei's genetic distance (1978), which was obtained via RAPD. Individuals in *Atta sexdens rubropilosa* populations collected from Ivatuba, Maringá, Presidente Prudente, Dracena, and Itambé were grouped with the UPGMA method using the Popgene 1.31 program.

DNA quantity is too low or high. Carvalho (2000) found that for *A. s. rubropilosa*, the optimum amount of DNA for RAPD reactions is between 19 and 30 ng. Our study shows that it was possible to extract high-quality DNA that ranged between 10 and 40 ng for amplification.

RAPD-PCR marker proved efficient for genetic populations studies of *A. s. rubropilosa*, because 148 fragments reproducible were obtained with 15 primers. Grutzmacheri *et al.* (2007) used three primers (UBC 354, UBC 348, and UBC356) for RAPD to study the genetic variability of the *Acromyrmex* genus collected from Rio Grande do Sul and obtained 87 fragments.

Leaf-cutting ants of the Atta genus are indigenous to the Americas. In the Americas, the distribution of these insects extends from the southern U.S. to northern Argentina, except for Chile and some of the Antilles islands (Sousa, 1996).

Originally, there were no *Atta* ants in the forests of Paraná. They colonized the state following the first reforestation event in Paraná during the 1940s (Bansho *et al.*, 1994).

In the short time since the introduction of *A. s. rubropilosa* ants in Paraná (approximately 70 years), they have obtained a high degree of differentiation (G_{ST} = 0.2372), but there is high variation within populations as shown by AMOVA. Chemical control of *A. s. rubropilosa* populations considered pests by farmers, should be careful. The high intra-population polymorphism and possible gene flow between populations may promote the occurrence of cross-resistance to pesticides used to control them.

Despite the short geographical distance (36.5 km) between Ivatuba and Itambé, these populations have the highest genetic distance (Table 2). It is possible to infer that these breeding populations do not mate or that there are a small number of hybridizations. Among the Maringá and Ivatuba populations (separated by 42.3 km), the lowest estimated genetic distance was observed (Table 3). However Mantel test (R = 0.344; p = 0.001) indicate that there is relationship between the genetic and geographic distances of the studied nests. These results support stepping-stone model proposed by Kimura (1953). This model assumes that species occupying a wide geographic area, forming differentiate geographical races (Kimura and Weiss, 1964). Analyzed *A. s. rubropilosa* populations do not constitute races, but can be considered subpopulations. The high genetic differentiation values may be due to the existence of an abundant ancestral polymorphism that can be maintained by multiple mating of the queen. The high polymorphism (83.11 %) indicates that this species of leaf-cutter ant is adapted to the region and integrated programs for the control should be developed.

CONCLUSIONS

Leaf-cutting ants' *A. s. rubropilosa* showed extensive polymorphism and genetic diversity that were higher within populations than between them. The values of genetic distance and geographic are not randomized and analyzed populations are moderately differentiated.

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