SYSTEMATICS, MORPHOLOGY AND PHYSIOLOGY

Isoenzymatic Polymorphism in the Leaf-Cutting Ant *Atta capiguara* Gonçalves (Hymenoptera: Formicidae)

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ABSTRACT - This study was carried out to analyze the genetic population structure of *Atta capiguara* from 12 nests collected in Tapejara in the state of Paraná, Brazil, using isoenzyme polymorphisms. The analyzed isoenzymes were esterases (EST – EC 3.1.1.1), acid phosphatase (ACP – EC 3.1.3.2) and carbonic anhydrase (CA – EC 4.2.1.1). Ten *loci* were found in *A. capiguara* and four polymorphic loci were detected. The observed heterozigosity (0.0296) was low when compared to the expected heterozigosity (0.1461). The high value of F_{IS} (0.7954) shows an excess of homozygous genotypes probably caused by inbreeding.

KEY WORDS: Isoenzyme, genetic variability, population genetics

Leaf-cutting ants are widely distributed from southern United States to central Argentina, causing heavy damage in agriculture and attacking a wide range of vegetables, ornamental plants, cultivated and reforested trees and pastures. The two genera (*Atta* and *Acromyrmex*) of leaf-cutting ants found in Brazil are very diverse in species (Augustin *et al* 1999).

There are 10 species of leaf-cuttings in the genus *Atta* in Brazil, but just five of them are of economic importance to agriculture (Forti 2000). Among them, we can mention *Atta capiguara* Gonçalves that attacks pastures and sugarcane. This species constructs diffuse nests, characterized by multiple tumuli of excavated soil dispersed over a dozen of square meters (Amante 1964), and it occurs mainly in three Brazilian states, São Paulo, Mato Grosso and Minas Gerais (Della Lúcia 1993).

Ants are haplodiploid social insects and constitute highly complex colonies with several morphological castes with different functions. They make an interesting model for studies on behavior ecology, population genetics, kin selection and altruism (Brian 1983, Höldobler & Wilson 1990). Although many studies on the physiology, morphology, ecology and control methods of leaf-cutting ants have been published, only insufficient information on their taxonomy is available. Tools that would allow for the identification of insects based on their genotype differences by using molecular markers such as isoenzymes would be really helpful in understanding the biological diversity in this group of insects. Therefore, the objective of this study was to analyze the genetic variability and population structure of leaf-cutting ants using isoenzymes in order to understand their genetic social structure.

Material and Methods

Ant samples. Workers from twelve nests of leaf-cutting ants *A. capiguara* were collected in Tapejara (23º 43' 59" S; 52º 52' 24" W) in the northwestern region of the state of Paraná, Brazil. Some of the collected insects were used for identification and others were maintained at -20ºC until isoenzyme analysis. Six worker ants from each nest were analyzed for three enzymatic systems. Samples were homogenized in 80 µl of 10% 2-mercaptoethanol and glycerol and then centrifuged at 16,000 g for 10 min at 2ºC. Supernatants were then individually loaded onto polyacrylamide and starch gels.

Esterase (EST – EC 3.1.1.1) electrophoresis analysis. Polyacrylamide gels (10%) were prepared in 0.375 M Tris-HCl, pH 8.8 (Lapenta *et al* 1995). From each prepared sample, 15 µl of supernatant were separated by electrophoresis (constant voltage 196 V, for 5h, at 4ºC). Running buffer consisted of 0.1 M Tris-glycine (pH 8.3) and staining for esterase activity was done according to Lapenta *et al* (1995). Gels were soaked for 30 min in 50 ml of 0.1 M sodium phosphate (pH 6.2) at room temperature. Esterase activity was visualized by incubating the gels for 1 h in a solution containing 50 ml 0.1 M sodium phosphate, 20 mg β-naphthyl acetate, 30 mg α-naphthyl acetate, 60 mg Fast Blue RR salt and 5 ml *n*-propanol. Substrate specificity was verified by staining the gels only with α-naphthyl acetate or β-naphthyl acetate separately and then comparing the result with that of gels stained with a mixture of α- and β-naphthyl acetate. In the case of long-term storage, gels were soaked for 1h at room temperature in a mixture of 7.5% acetic acid and 10% glycerol containing 5% gelatin (Ceron *et*

al 1992). They were then placed between two tightly stretched sheets of wet cellophane paper and dried for 24-48h.

Acid phosphatase (ACP – EC 3.1.3.2) and carbonic anhydrase (CA – EC 4.2.1.1) electrophoretic analysis. Horizontal technique on 14% starch gels (Penetrose 30, Corn Products of Brazil S.A.) (Smithies 1955) was used for electrophoretic analysis of acid phosphatase and carbonic anhydrase (ACP). Supernatants from samples initially homogenized were then individually loaded onto the gel.

Detection of ACP activity was accomplished with 0.5ml magnesium chloride 1 M, 0.5 ml manganese chloride 0.1 M, sodium acetate buffer 0.05M pH 5.5 and 0.04g Fast Blue RR Salt and 30 mg α-naphthyl-phosphate 1% (Alfenas 1998).

Carbonic anhydrase reacts with a substrate forming a non stratified fluorescent product. Fluorescein diacetate (10mg) was dissolved in 0.5 ml acetone and mixed in 100 ml of 0.1 M sodium phosphate buffer, pH 6.5. The gel was soaked in the solution, incubated at 37°C for 40 min and observed under long wave ultraviolet light (240 nm).

POPGENE software (Yeh *et al* 1999) was employed to analyze genetic population structure of *A. capiguara*. Polymorphic loci were submitted to *chi*-square tests to determine the deviations from expected genotypic frequency according to the Hardy-Weinberg's equilibrium. Inbreeding coefficient (F_{IS}) was evaluated by F-statistics (Wright 1984).

Results and Discussion

Analysis of the three isoenzymes (acid phosphatase, carbonic anhydrase and esterase) identified 10 loci in the leaf-cutting *A. capiguara* (Fig 1), four of which were polymorphic. Since the estimate of polymorphic loci was high for Hymenoptera, it is necessary to take into account the small number of analyzed loci. The three enzymatic systems analyzed in this study are important for detection of molecular markers that can be used as bioindicators for pesticides.

Theoretical models based on neutrality and on selection predict that the probability of genetic polymorphism is smaller in haplodiploid populations than in diploid ones. When heterozygous absence occurs in one of the sexes, selection will influence the fixation of alleles. Stochastic processes or random drift possess a similar effect (Mayo 1976). It has been demonstrated that the level of genetic variability of isoenzymes is really smaller in Hymenoptera species than in diploid insects (Pamilo *et al* 1978).

Loci $Acp\text{-}2^{A}$ (0.9048) and $Ca\text{-}1^{C}$ (0.0423), respectively, showed the higher and lower allelic frequency in *A. capiguara* (Table 1). *Atta capiguara* presented six esterase loci. All esterase loci analyzed were monomorphic. The lack of genetic variability for the esterases in ants has already been reported by Pamilo *et al* (1975) and Augustin *et al* (1999).

Average heterozygosity (0.0296) in *A. capiguara* was very low when compared to the expected heterozygosity (0.1461) (Table 2).

Mean differences between observed and expected heterozygosity may be explained partly by the small number of nests and/or of loci analyzed (Table 2). Pamilo *et al* (1978) obtained similar results in a study on 13 *Formica* species. Although total diversity (average heterozygosity) obtained by the above-mentioned authors was 0.016 (± 0.014), it varied among populations and depended on the number of loci analyzed and nests studied.

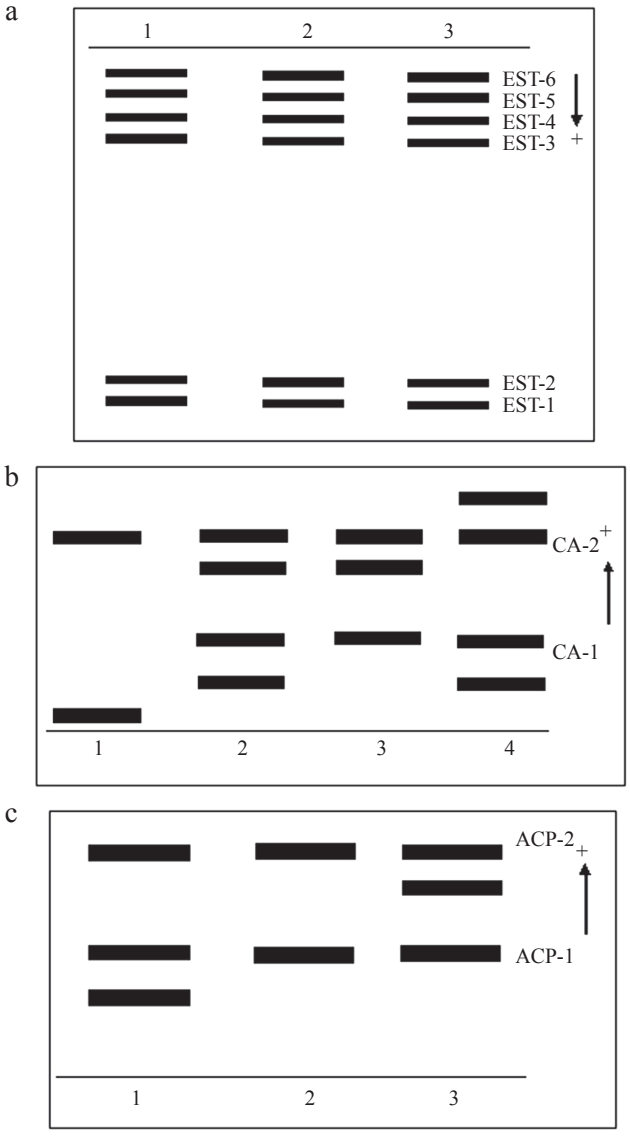

Fig 1 Zymogram of *Atta capiguara* isozymes. a) = esterase; b) = carbonic anhydrase; c) = acid phosphatase.

Table 1 Isozyme allelic frequency in *Atta capiguara*.

Loci	*Atta capiguara*		
	Allele A	Allele B	Allele C
Acp-1	0.8629	0.1371	-
Acp-2	0.9048	0.0952	-
Ca-1	0.4154	0.5846	0.0423
Ca-2	0.5513	0.3718	0.0769

Table 2 Isozyme Mean Heterozygosity for 10 loci analyzed in *Atta capiguara.*

Loci	*Atta capiguara*		
	Sample size	Obs. Het.	Exp. Het.
Acp-1	62	0.0454	0.2385
Acp-2	42	0.0476	0.1444
Ca-1	65	0.1231	0,4894
Ca-2	39	0.0769	0.5591
Est-1	72	0.0000	0.0000
Est-2	72	0.0000	0.0000
Est-3	72	0.0000	0.0000
Est-4	72	0.0000	0.0000
Est-5	72	0.0000	0.0000
Est-6	72	0.0000	0.0000
Mean	64	0.0296	0.1461
St. Dev		0.0433	0.2175

Low values obtained for heterozygosity in ants may also be explained by the theory of kin selection (Hamilton 1964 a,b), or rather, haplodiploidy promotes an asymmetry in gene transmission with a decrease in variability.

The genotypes obtained with the three analyzed systems showed that *A. capiguara* nests were monogynic. However, few isozymes were analyzed and the queen was not looked for in the nests. *Atta* nests are monogynic, just *Atta texana* found in Mexico, is described as polygynic (Diehl *et al* 2001). The fixation index (F_{IS}) for *A. capiguara* was high (0.7954) (Table 3). This result shows that inbreeding is occurring in that population.

It seems that the genetic structure of ant populations varies widely between species and populations of the same species, depending on the number of nest founders (monogyny, polygyny, monoandry, polyandry).

In many ant species, nests are secondarily polygynic. In other words, the daughters of the resident queen are recruited to return to the original colony (Chapuisat & Keller 1999) and new nests may be established and be dependent of "budding" (formation of new nests starting from the oldest one) (Gyllenstrand & Seppä 2003).

Table 3 Fixation Index (F_{IS}) for 10 loci of isozymes analyzed in *Atta capiguara.*

Loci	Sample size	F_{IS}
Acp-1	124	0.7955
Acp-2	84	0.7237
Ca-1	130	0.7466
Ca-2	78	0.8606
Est-1	144	****
Est-2	144	****
Est-3	144	****
Est-4	144	****
Est-5	144	****
Est-6	144	****
Mean	128	0.7954

Microsatellite analysis of populations of the ant *Rhytidoponera metallica* Smith showed that very closely located nests were not more genetically similar than those farther away. This was a surprising fact since the new colonies were formed by "budding" and dispersion was restricted (Chapuisat & Crozier 2001).

The social way of life and climate stability may explain genetic uniformity in Hymenoptera populations with possible low genetic diversity levels in ants (Pamilo *et al* 1975). However, haplodiploidy does not seem to be the only explanation for reduced diversity in Hymenoptera. Nest stability may be related to its permanence, since regulation capacity seems to be weak in small nests (Pamilo *et al* 1978). Therefore, the maintenance of the nest is related to environmental stability due to the fact that a great amount of energy is necessary to build and maintain the colony (Rosengren 1971). Another reason that alters ant diversity is the fact that they have low dispersal, in other words, when alates leave to mate, they do not fly far from their nests and this promotes inbreeding. The second factor would be the occurrence of secondary polygyny in the species. This mechanism of nest division has been frequently detected in ants and has been sufficiently discussed by several authors (Trunzer *et al* 1998, Hora *et al* 2005, Kellner *et al* 2007). This fact may have occurred due to females restricting dispersion by secondary polygyny and, consequently, the new nests were dependent on the founder nest. Such characteristic may assume a strong genetic differentiation, as has been shown in several ant species (Diehl *et al* 2001, Gyllenstrand & Seppä, 2003, Mäki-Petäys *et al* 2005).

The study of populations of *Formica* in fragmented areas showed that genetic studies may be reliably used to analyze recent historical changes underlining population genetic structure, and that species with different social structures may respond differently to habitat changes (Mäki-Petäys *et al* 2005).

Ants have unique characteristics that are important for assessing the vulnerability of their populations. The ratio between effective population size and ant biomass is low, the social structure may restrict the dispersion of individual ants promoting a spatial differentiation (Seppä & Pamilo 1995), and the sex determination system leads to an inbreeding depression (Cook & Crozier 1995). Fragmentation of habitat causes dangerous consequences in all these respects, but the population changes occur slowly because the length of each generation is usually very long (Hölldobler & Wilson 1990) and the colonies can remain alive for a very long time.

The *A. capiguara* population from Tapejara showed low heterozygosity for the analyzed isoenzymes. This fact could be due to inbreeding or caused by secondary polygyny, or be a result of the small number of nests analyzed. However, further studies are needed with molecular markers increasing the population size and the number of analyzed loci.

The low genetic variability detected for isoenzymes,

especially esterases, may contribute to studies where leaf-cutting ants can be used as bioindicators for pesticides in agriculture or for its control when they become pests. Alterations in the esterase expression profile can be detected, indicating the presence of pesticide residues. In the case of pest control, up or down regulation of the expression or abundance of esterases that were not synthesized before in a monitored population can be detected and evaluated by molecular techniques.

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