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Mitochondrial DNA variability in populations of *Centris aenea* (Hymenoptera, Apidae), a crop-pollinating bee in Brazil

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ABSTRACT. *Centris* spp are oil-collecting solitary bees that are valuable pollinators of crops such as Brazil nut, cashew, and acerola. We investigated the genetic variability of populations of *C. aenea* in the northeastern region of Brazil. Total DNA was extracted from 59 individuals from 6 locations in the States of Pernambuco and Bahia and a 600-650-bp fragment of the mitochondrial COI/COII region amplified by PCR, followed by digestion with the restriction enzymes *Dra*I and *Ssp*I. PCR-RFLP analysis revealed eight different haplotypes among the populations. Haplotype A1, revealed by *Dra*I, was the most frequent (50%), and haplotypes A3 and A4 were exclusive to Feira de Santana, Bahia and Morro do Chapéu, Bahia, respectively. Among the

haplotypes revealed by *Ssp*I, B2 was the most frequent (37%) and B3 was exclusive to Feira de Santana. This information revealing high haplotype diversity will be useful for developing management strategies for *Centris*, especially because of increasing interest in the rearing and/or relocation of these bees for crop pollination.

Key words: Centridini; PCR-RFLP; Population variation; Solitary bee

INTRODUCTION

Recent studies on bee fauna in orchards have indicated that *Centris aenea* females are frequent visitors of cultivated plants such as acerola (*Malpighia emarginata*) and guava (*Psidium guajava*), contributing to improvement of productivity and fruit quality (Freitas et al., 1999; Berti-Boti, 2001; Guedes et al., 2011; Siqueira et al., 2011; Vilhena et al., 2012). Based on the extensive distribution of crops pollinated by *C. aenea* in Brazil (for instance, acerola is cultivated from the State of Rio Grande do Sul in the southern region of the country to the State of Ceará in the northeastern region) (Freitas et al., 1999; Carvalho, 2003) and on the possibility of relocating bees between different geographic regions to provide additional pollinators, studies on population variation have become of paramount importance.

Species in the genus *Apis* are the best studied among the Apoidea, for which there is a large body of literature on genetic variation that have helped studies on other bee groups as a parameter of comparison (Diniz et al., 2003; Francoy et al., 2009; Ting et al., 2009; Ivanova et al., 2010; Ji et al., 2011).

Relatively few genetic studies have investigated bee species within other genera, such as *Plebeia remota* (Francisco et al., 2001), *Eufrisea violacea* (Sofia et al., 2005), *Macrotera portalis* (Danforth et al., 2003), *Tetragonisca angustula* (Oliveira et al., 2004), *Partamona helleri* (Brito and Arias, 2010), *Partamona seridoensis* (Fernandes et al., 2012), *Melipona subnitida* (Cruz et al., 2006), and *Euglossa fimbriata* (Suzuki et al., 2010). Some of these studies have used PCR-RFLP (Francisco and Arias, 2010; Brito and Arias, 2010; Suzuki et al., 2010) revealing interesting information that has allowed researchers to distinguish populations. The COI/COII mitochondrial DNA region is known to be highly variable in *Apis mellifera* (Garnery et al., 1993; Franck et al., 1998) and stingless bees (Francisco et al., 2001; Fernandes-Salomão et al., 2002; Weinlich et al., 2004; Moretto and Arias, 2005; Brito and Arias, 2005).

The only information available on population variation of *C. aenea* is a morphometric study (Ferreira et al., 2011), which revealed low population variation. To address this lack of information about the genetic variation in this species, we investigated the levels of genetic differentiation between populations of *C. aenea* in the northeastern region of Brazil using PCR-RFLP in the COI/COII mitochondrial DNA region.

MATERIAL AND METHODS

Females (N = 59) of *C. aenea* were collected from six localities in the States of Bahia and Pernambuco, in the northeastern region of Brazil (Tables 1 and 2). Sampled areas included natural vegetation and acerola orchards usually visited by *C. aenea* (Table 1). Specimens were

Table 1. Number of *Centris aenea* females, sampled localities, geographical coordinates, and number of individuals used in the PCR-RFLP analysis (modified from Ferreira et al., 2011).

Locality	Vegetation	State	Latitude	Longitude	N	PCR/RFLP
Feira de Santana	Caatinga	Bahia	12º 11' 58.3"	38º 58' 05.95"	10	3
Mucugê	Campo rupestre	Bahia	12º 50' 45"	41º 31' 04"	9	2
Morro do Chapéu	Caatinga	Bahia	11º 34' 29"	41º 11' 00"	10	2
Palmeiras	Cerrado	Bahia	12º 32' 44"	41º 43' 31"	10	1
Cruz das Almas	Acerola orchard	Bahia	12° 40' 39"	39° 40' 23"	10	5
Petrolina	Acerola orchard	Pernambuco	09º 09'	40º 22'	10	5

collected using an entomological net, fixed with ethyl acetate, and preserved in 100% ethanol. Voucher specimens were deposited at the Coleção Entomológica Prof. Jonhan Becker of the Museu de Zoologia da Universidade Estadual de Feira de Santana (MZUEFS).

Total nucleic acid extractions from the thorax of alcohol-preserved individuals followed three protocols: 1) phenol-chloroform modified from Raeder and Broda (1985), with the addition of 5 µL 20 µg/mL proteinase K before incubation and non-simultaneous addition of phenol and chloroform; 2) phenol-chlorophorm by Han and McPheron (1997), and 3) the "DNeasy Blood and Tissue kit" (QIAGEN). A fragment of 600-650 bp within the COI/COII mitochondrial region was amplified by polymerase chain reaction (PCR) using primers 5'-TCTATACCACGACGTTATTC-3' and 5'-GATCAATATCATTGATGACC-3' (Hall and Smith, 1991). PCRs were carried out in 25 µL according to Gasparich et al. (1995), using 2 µL genomic DNA. The cycle program consisted of an initial denaturation step of 5 min at 94°C, followed by 40 cycles of 1 min at 94°C, 1 min at 44° or 45°C, 2 min at 72°C, with a final extension step of 10 min at 70°C.

PCR products were visualized on ethidium bromide-stained 1% agarose gels in 1X TBE buffer, along with a 100-bp ladder (GE Healthcare).

PCR products of 18 individuals were digested with *Dra*I and *Ssp*I (Table 1).

The digestion reaction mixture was prepared in a final volume of 20 µL, using 2 µL 10X buffer, 1 U *Dra*I or *Ssp*I; 10.8 µL milliQ water, and 7 µL PCR product and incubated at 37°C for 12 h. Digestion products were visualized by electrophoresis on 2-3% ethidium bromide-stained agarose gels using a 100-bp ladder to estimate fragment size.

Median-joining networks (Bandelt et al., 1999) were reconstructed for mitochondrial haplotypes, with the Network version 4.5.0.0 program (www.fluxus-engineering.com).

RESULTS

Digestion of the amplified fragment of the COI/COII region with *Dra*I revealed four restriction patterns (Figure 1): haplotype A1, uncut fragment (600 bp), haplotype A2 (390 and 180 bp fragments), haplotype A3 (350 and 140 bp fragments) and haplotype A4 (400 and 230 bp fragments).

Digestion with *Ssp*I also generated four restriction patterns (Figure 1): haplotype B4, uncut fragment (600 bp), haplotype B1 (300 and 190 bp fragments), haplotype B2 (265 and 190 bp fragments), haplotype B3 (265 and 210 bp fragments).

Among the haplotypes revealed by digestion with *Ssp*I, haplotype B1 was found in samples from Cruz das Almas and Petrolina, which are 399 km apart (Table 2). Haplotype

B2 was the most frequent (37%) and detected in Feira de Santana and in two localities in the Chapada Diamantina (Mucugê and Morro do Chapéu), whereas haplotype B4 was found in Feira de Santana and Petrolina, which are 394 km apart. Haplotype B3 was exclusive of Feira de Santana (Table 2).

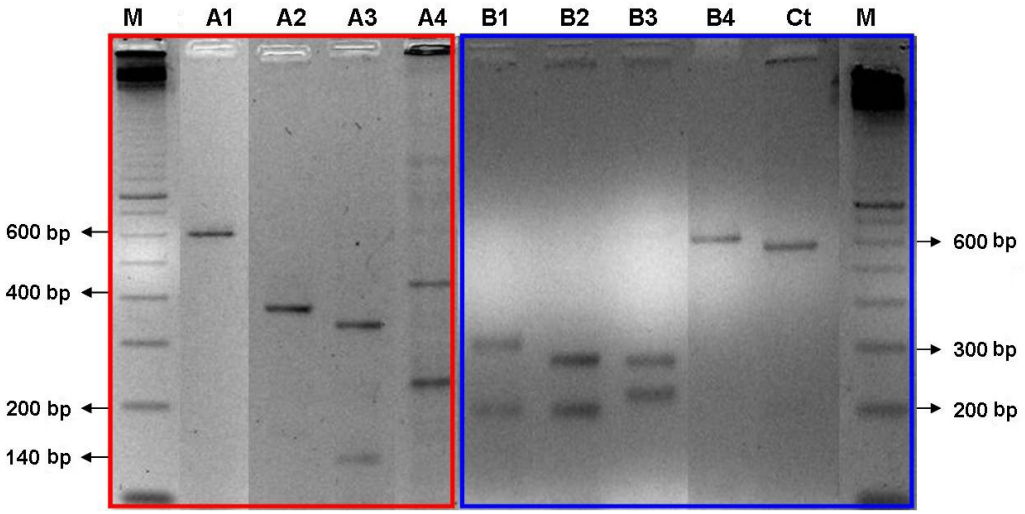

Figure 1. Two percent agarose gel (red) and 3% (blue) stained with 25 µg/µL ethidium bromide showing 8 lanes with the haplotypes identified in 6 populations of *Centris aenea*. *Lane M* = 100-bp ladder (GE Healthcare); *lanes A1*, *A2*, *A3*, and *A4* = *Dra*I haplotypes; *lanes B1*, *B2*, *B3*, and *B4* = *Ssp*I haplotypes; *lane Ct* = control with non-digested PCR product.

Table 2. Distance in km among localities sampled based on latitude and longitude.

	FSA	MU	MC	PE	PAL
MU	277.8				
MC	256.4	145.9			
PE	394.2	429.7	284		
PAL	296.9	175.1	123	405.7	
CA	78.82	200.9	204.9	399.5	223.2

FSA = Feira de Santana; MU = Mucugê; MC = Morro do Chapéu; PE = Petrolina; PAL = Palmeiras; CA = Cruz das Almas (modified from Ferreira et al., 2011).

Among the haplotypes revealed by digestion with *Dra*I, haplotype A1 was the most frequent (50%) and was detected in 5 of the 6 populations; it was absent only in the three samples from Feira de Santana. Haplotype A2 was detected in three populations (Feira de Santana, Mucugê, and Petrolina) in 30% of all samples analyzed. Haplotypes A3 and A4 were each exclusive of one population, Feira de Santana and Morro do Chapéu, respectively. In the three localities sampled in the Chapada Diamantina (Palmeiras, Morro do Chapéu, and Mucugê), three distinct haplotypes were detected (A1, A2, and A4) (Table 3).

The population of Feira de Santana had the highest haplotype diversity (five) in only three individuals studied and also the largest number of exclusive haplotypes (A3 and B3) (Table 3).

Table 3. *Dra*I and *Ssp*I haplotypes identified 6 populations of *Centris aenea*.

Haplotypes	Locality					
	CA (5)	FSA (3)	MC (2)	Mu (2)	Pe (5)	Pal (1)
A1	*		*	*	*	*
A2		*		*	*	
A3		*				
A4			*			
B1	*				*	
B2		*	*	*		
B3		*				
B4		*			*	

CA = Cruz das Almas; FSA = Feira de Santana; MC = Morro do Chapéu; Mu = Mucugê; Pe = Petrolina; Pal = Palmeiras. Numbers in parentheses represent the number of individuals per locality.

The haplotype network (Figure 2) shows evidence of genetic polymorphism in populations of *C. aenea* and some genetic structuring in the populations from Cruz das Almas, Petrolina, Feira de Santana, and Mucugê, which formed distinct haplogroups (Figure 2).

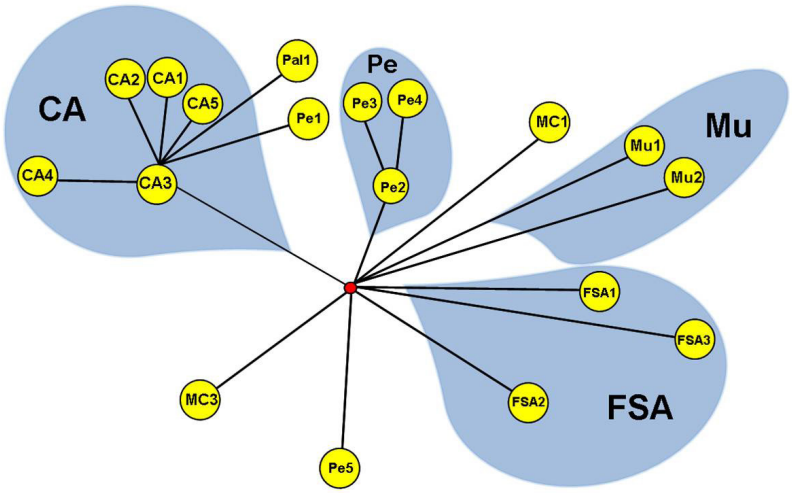

Figure 2. Median-joining network diagram for *Dra*I and *Ssp*I haplotypes. The branch length corresponds to the genetic distance between haplotypes. CA = Cruz das Almas; Pal = Palmeiras; Pe = Petrolina; MC = Morro do Chapéu; Mu = Mucugê; FSA = Feira de Santana.

DISCUSSION

The size of the amplified COI/COII fragment of *C. aenea* (600-650 bp) was similar to what has been found for *Melipona* species (approximately 630-650 bp) (Fernandes-Salomão et al., 2002) and shorter than the same region in *Partamona* species (929 bp) (Brito and Arias, 2005). In *A. mellifera* subspecies, the COI/COII region was considerably longer and had variable length due to up to 450 bp in the intergenic region (noncoding region between the RNAtLeu and COII genes), which normally ranges from 200 to 650 bp (Cornuet and Garnery, 1991). Sequencing of the mitochondrial genome of *Melipona bicolor* (Silvestre et

al., 2008) confirmed that the intergenic region is not present in *Melipona*, *Plebeia* (Francisco et al., 2001), or *Partamona* (Brito and Arias, 2005). We amplified the COI/COII region in *C. aenea* with the same primers used for *A. mellifera* and *Melipona* species, which suggests that *C. aenea* also lacks the intergenic region, as do the meliponine bees (Francisco et al., 2001; Brito and Arias, 2005). However, it is still necessary to sequence this fragment in *C. aenea* and compare it with the available sequences of other bee species, since presence or absence of an intergenic region in the COI/COII fragment can explain a substantial part of the genetic variation observed in different bee genera. In contrast with a morphometric study that showed low population variation in *C. aenea* (Ferreira et al., 2011), we found intra- and interpopulational variation in *C. aenea* based on the 8 haplotypes identified thus far.

The haplotype diversity observed (8 haplotypes, with 2 restriction enzymes in 6 populations) in the *C. aenea* populations that we sampled was higher than that found for the social bee *Partamona helleri* (10 haplotypes, with 6 restriction enzymes, in 11 populations) (Brito and Arias, 2010).

Interpopulational divergence in *C. aenea* shown in the median-joining network was found between both geographically close (Feira de Santana and Cruz das Almas, 78.82 km) and more distant populations (Cruz das Almas and Petrolina, 399.5 km apart). Four haplogroups were defined: Cruz das Almas, Petrolina, Feira de Santana, and Mucugê, without direct relationship with their relative geographic locations (Table 2; Figure 2). Feira de Santana and Cruz das Almas were the closest sampled locations (78.82 km) (Table 2); yet their haplogroups occupied opposite positions (Figure 2). Moreover, branch length indicated a relatively great genetic distance between the haplotypes from these two locations. Our results suggest population structure due to four distinct haplogroups.

The observed distribution of haplotypes among the *C. aenea* populations that we studied might be related to the fragmentation and/or reduction of its original distribution range and the influence of environmental barriers inadequate for colonization by this bee. *C. aenea* has some environmental requirements for the maintenance of its populations, including a need for oil-producing plants, which can impose barriers to gene flow. This seems to be a plausible explanation, since, with rare exceptions, *Centris* species are known to depend on floral oil-producing plants such as species in the families Malpighiaceae and Krameriaceae, for building their nests and/or feeding their larvae (Gaglianone, 2003; Aguiar and Garófalo, 2004; Aguiar et al., 2006). Ramalho and Silva (2002) found that in a restinga environment in the Brazilian Northeast, the population abundance of Centridini was proportional to the abundance of oil-producing plants. These plants, in turn, are abundant in areas with natural vegetation but are not common in anthropized areas.

The management of bees, including *C. aenea*, can be an alternative to the crisis in ecosystem services of crop pollination caused by burning, introduction of competing species, habitat fragmentation, and pesticides (Nabhan and Buchmann, 1997). It could also be an interesting alternative for Brazilian agriculture, which is highly dependent on crop pollination services on a large scale provided by a single bee species (*A. mellifera*). The rearing, relocation, and release of solitary bees, such as *C. aenea*, into orchards can be feasible in Brazil, as it is already an established practice in other countries, using other species of solitary bees (Bosch et al., 2002).

Further studies regarding genetic variation within *C. aenea* should be carried out with other molecular markers and a broader sampling. Such studies coupled with investigation on

flight range and reproductive biology of this species (such as preferred mating sites and dispersion ability of males and virgin females) can help us to better understand variation in *C. aenea* populations.

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