Short Communication

Very low mitochondrial variability in a stingless bee endemic to cerrado

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Abstract

Partamona mulata is a stingless bee species endemic to cerrado, a severely threatened phytogeographical domain. Clearing for pasture without proper soil treatment in the cerrado facilitates the proliferation of termite ground nests, which are the nesting sites for *P. mulata*. The genetic consequences of these changes in the cerrado environment for bee populations are still understudied. In this work, we analyzed the genetic diversity of 48 colonies of *P. mulata* collected throughout the species' distribution range by sequencing two mitochondrial genes, cytochrome oxidase I and cytochrome B. A very low polymorphism rate was observed when compared to another *Partamona* species from the Atlantic forest. Exclusive haplotypes were observed in two of the five areas sampled. The sharing of two haplotypes between collection sites separated by a distance greater than the flight range of queens indicates an ancient distribution for these haplotypes. The low haplotype and nucleotide diversity observed here suggests that *P. mulata* is either a young species or one that has been through population bottlenecks. Locally predominant and exclusive haplotypes (H2 and H4) may have been derived from local remnants through cerrado deforestation and the expansion of a few colonies with abundant nesting sites.

Keywords: cerrado, mtDNA, genetic variability, *Partamona mulata*.

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The land area cleared for pasture in Brazil has increased markedly in the past 40 years and has been driven largely by the beef industry (IBGE, 1974, 2010). Considerable clearance of natural vegetation has occurred in the cerrado. This phytogeographical domain is characterized by a variety of different vegetation physiognomies ranging from grassland to tall woodland, with well-defined seasons that include a very dry season (April to November), when bushfires are common, followed by a humid, rainy season (December to March), when vegetation blossoms (Batalha *et al.*, 2001, 2011). Only 20% of the original cerrado remains and the endemic vegetation and fauna are under constant threat because of systematic deforestation to provide land for crops or livestock (Myers *et al.*, 2000). Damage to this environment is a threat to bees because it reduces the availability of nesting sites and isolates populations into small fragments and this may lead to inbreeding depression (Kerr *et al.*, 1996). The genetic consequences of these changes for bee populations endemic to the cerrado are still poorly understood.

Habitat fragmentation in the cerrado has been shown to increase inbreeding in populations of the stingless bee *Melipona rufiventris* in Minas Gerais state, Brazil (Tavares *et al.*, 2007). A low genetic variability of microsatellite markers has also been observed in 59 nests of *P. mulata* from five collection sites in cerrado areas of the states of Mato Grosso and Mato Grosso do Sul (Francisco *et al.*, 2006). In the latter case, the microsatellite loci were genotyped using heterospecific primers and the low genetic variability was considered to be an artefact of null alleles rather than a true status for the species.

Although deforestation is usually claimed to be a major cause of reductions in bee populations (Kearns *et al.*, 1998; De la Rúa *et al.*, 2007; Tavares *et al.*, 2007), in the cerrado deforestation together with inadequate soil treatment contributes to the budding of termite ground nests, thereby increasing the availability of nesting sites for *Partamona* bees. *Partamona* is a Neotropical stingless bee genus of the tribe Meliponini, distributed from southern Mexico to southern Brazil (Camargo and Pedro, 2003). Except for *Partamona helleri*, which usually uses abandoned bird nests and epiphyte roots, most *Partamona* species build their nests in active termite nests. *Partamona mulata* is a termitophile species endemic to central Brazil (Mato Grosso do Sul and Mato Grosso) and Bolivia and is always associated with ground termite mounds in the cerrado (Pedro and Camargo, 2003).

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The analysis of mitochondrial DNA (mtDNA) polymorphisms is a useful approach for investigating genetic variability and has been successfully applied to studies of insect populations, including Meliponini species (Queza-da-Euán *et al.*, 2007; Francisco and Arias, 2010). In this work, we used the polymorphism of two mitochondrial genes to investigate the genetic status of *Partamona mulata,* a highly endemic cerrado species, and make inferences about the historical events that could explain the observed haplotype distribution.

We analyzed one bee from each of 48 natural nests of *P. mulata* throughout the species' distribution range in cerrado areas of central western Brazil: Cuiabá (Cba; n = 13), Santo Antônio do Leverger (Sal; n = 4), Poconé (Poc; n = 13), Cáceres (Cac; n = 6) and Campo Grande (Cgd; n = 12) (Figure 1). The bees were stored at -80 °C prior to DNA extraction. All specimens were identified by Dr. Silvia Regina de Menezes Pedro (Faculdade de Filosofia, Ciências e Letras de Ribeirão Preto, USP - FFCLRP/USP) and voucher specimens were deposited in the collection of the Department of Biology at FFCLRP/USP.

Total DNA was extracted from one thorax per tube using the Chelex® (Bio-Rad) protocol (Walsh *et al.*, 1991). For mtDNA amplification, universal primers for insects (Simon *et al.*, 1994) were used to amplify 451 bp of the cytochrome oxidase I (*CO*I) gene (mtD6+mtD9) and 402 bp of the cytochrome B (*Cyt*B) gene (mtD26+mtD28). PCR assays were done in a final volume of 50 μL containing 4 μL of DNA, 5 μL of 10x PCR buffer, 1 μL of each primer (20 μM), 5 μL of dNTPs (2 mM each), 1.5 μL of 50 mM MgCl$_2$ and 2.5 U of *Taq* DNA polymerase (Invitrogen). The PCR cycling conditions were as described elsewhere (Brito and Arias, 2005). Aliquots of the PCR

products were run on 1% agarose gels for quantification, stained with GelRed (Biotium) and photographed under a UV transilluminator. The remaining PCR products were purified by digestion with 0.5 μL of ExoSAP-IT® (USB) and sent to Macrogen, South Korea, for sequencing in an automatic sequencer 3730*xl* (Applied Biosystems, USA). Electropherogram visualization, alignments and concatenation were done using the program Geneious v.5.1.6 (Drummond *et al.*, 2010). The sequences obtained were deposited in GenBank.

Sequences obtained from the *CO*I (accession numbers: JX480903 to JX480950) and *Cyt*B (accession numbers: JX480951 to JX480998) genes were concatenated in an 853 bp sequence for all samples. Haplotypes were analyzed using DnaSP v.5 (Librado and Rozas, 2009) to calculate haplotype diversity (*Hd*) and nucleotide diversity (π) (Nei, 1987). Exact tests (Raymond and Rousset, 1995) were calculated using Arlequin v.3.5 (Excoffier and Lischer, 2010) to estimate the genetic differentiation between pairs of populations. A haplotype network was generated by the median-joining network method (Bandelt *et al.*, 1999) using the software Network v.4.5.0.0.

The base composition of the 853 bp concatenated sequences of the *CO*I and *Cyt*B genes was A: 32.8%, C: 14.2%, G: 8.1% and T: 44.9%. No insertion/deletion (indels) polymorphisms were detected and only three sites were variable, with two transitions and one transversion. Consequently, the average nucleotide diversity was quite low (π = 0.00137). Two silent mutations were observed in *Cyt*B at the third base of codons; only one mutation was observed in *CO*I. The lower mutation rate in *CO*I was expected since this gene has higher functional restriction than *Cyt*B (Meiklejohn *et al.*, 2007). The mutation in *CO*I oc-

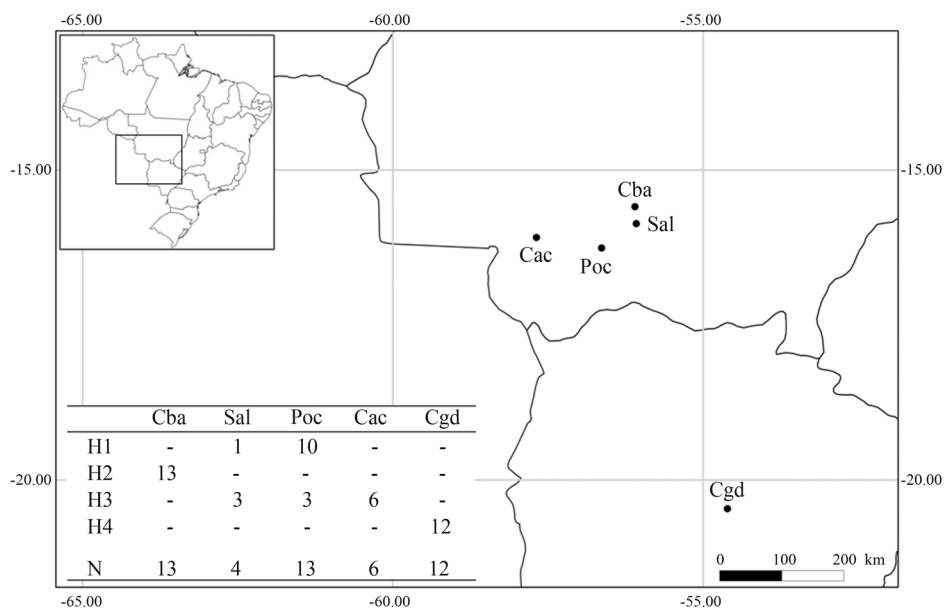|	Cba	Sal	Poc	Cac	Cgd
H1	-	1	10	-	-
H2	13	-	-	-	-
H3	-	3	3	6	-
H4	-	-	-	-	12
N	13	4	13	6	12

Figure 1 - Collection sites of *Partamona mulata* in cerrado areas in central Brazil: Cuiabá (Cba), Santo Antônio do Leverger (Sal), Poconé (Poc), Cáceres (Cac) and Campo Grande (Cgd). Haplotype frequencies are shown for each collection site.

curred at the first position and resulted in a conservative amino acid substitution (Val-Ile), *i.e.*, both amino acids were non-polar and aliphatic.

Only four haplotypes were observed in *P. mulata* and differed by one or two nucleotide substitutions. Figure 1 shows the haplotype occurrence and sharing between collection sites. Haplotype H1 was shared between Sal and Poc and H3, the most widespread haplotype, occurred at Sal, Poc and Cac. Haplotypes H2 and H4 were exclusive to the Cba and Cgd collection sites, respectively (Figure 1). Haplotype diversity (*Hd*) calculated for all collection sites together was 0.765; for individual collection sites, *Hd* varied from 0.000, where only one haplotype was observed (Cba, Cac and Cgd), to 0.500 (Sal).

Population differentiation values were significant ($p < 0.05$) but exceptions were observed between collection sites Cac/Sal and Poc/Sal. The haplotype network showed H3 positioned in the centre with all other three haplotypes distant from it by a single mutation step (Figure 2).

Unlike other Meliponini genera that are commercially exploited because of their high quality honey and by-products, *Partamona* is not harvested and consequently not transported by beekeepers, probably because of this genus's anti-hygienic habit of collecting animal faeces (personal observation). This fact led us to discard the hypothesis that haplotype sharing was due to human interference. In addition, *Partamona* queens are philopatric, meaning that they do not move more than 300 m from the maternal nest when they swarm (Wille and Orozco, 1975). Consequently, haplotype sharing by gene flow involving female *P. mulata* is also very unlikely since Poc is 100 km from Sal and Cac is almost 300 km from the other two sites (Figure 1).

Haplotype sharing in *P. mulata* is probably explained by ancient expansion of this species in the cerrado. Palynological and geological records indicate that, in the past, coastal areas of Brazil experienced greater modifications in vegetation cover than the central part of the country (Ab'
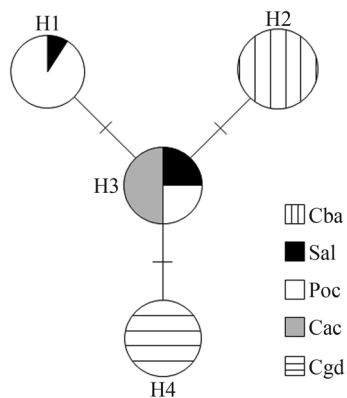

Figure 2 - Media joining network of concatenated partial sequences of the *CO*I and *Cyt*B mtDNA genes of samples of *Partamona mulata* showing the relationships among the haplotypes identified in this work. Cac - Cáceres, Cba - Cuiabá, Cgd - Campo Grande, Poc - Poconé and Sal - Santo Antônio do Leverger.

Saber, 1979; Carnaval and Moritz, 2008; Carnaval *et al.*, 2009). Thus, while the Atlantic forest contracted and many isolated fragments of vegetation emerged on mountain tops, in cerrado areas woodlands were replaced by grasslands that expanded during cooler periods (Amorim and Pires, 1996).

The low polymorphism rate observed here could be a consequence of small sampling size. However, a study of *P. helleri* that used a similar sample size (n = 47) to ours but a less sensitive marker (PCR-RFLP) identified 10 haplotypes in five sub-populations in the Brazilian Atlantic forest (Brito and Arias, 2010). Since these two species differ markedly in their distribution ranges, with *P. mulata* occupying a smaller area than *P. helleri* (Camargo and Pedro, 2003), a lower polymorphism rate would be expected for *P. mulata*. Other explanations for the low genetic variability observed here include the possibility that *P. mulata* is a young species or that it has experienced population bottlenecks in the recent past. According to Avise (2000), a star-like network is evidence of a species' expansion from a small number of founders. This phenomenon has been reported in natural populations of Hymenoptera, such as the ants *Diacamma indicum* (Viginier *et al.*, 2004) and *Formica cinerea* (Goropashnaya *et al.*, 2004) and the bees *Bombus insularis* (Estoup *et al.*, 1996), *Apis cerana* (Sihanuntavong *et al.*, 1999) and *Melipona rufiventris* (Tavares *et al.*, 2007).

Our data suggest that H3 is the most ancient of the observed haplotypes since it was present at three collection sites and was located in the centre of the network (Figure 2). Population expansion from a center of dispersion and the accumulation of mutations in mtDNA probably gave rise to the other haplotypes. A similar historical pattern has been postulated for stingless bees of the genus *Melipona* (Tavares *et al.*, 2007; Batalha-Filho *et al.*, 2010) and for the plant species *Caryocar brasiliense* and *Hymenaea stigonocarpa* (Collevatti *et al.*, 2003; Ramos *et al.*, 2007). The exclusive haplotypes can also be interpreted as local remnants after bottlenecks. However, given the fact that nucleotide divergence among the four haplotypes was very low, the first explanation seems to be more likely, *i.e.*, dispersion from a central location followed by mutations in mtDNA.

Partamona mulata is currently undergoing successful opportunistic expansion because of the abundance of nesting sites in termite nests. Future samplings over a broader area will be very helpful in understanding the correlation between cerrado degradation and *P. mulata* population expansion, as well as the increase in nest density.

Acknowledgments

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