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Genetic variability in *Melipona quinquefasciata* (Hymenoptera, Apidae, Meliponini) from northeastern Brazil determined using the first internal transcribed spacer (ITS1)

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ABSTRACT. *Melipona quinquefasciata* is a ground-nesting South American stingless bee whose geographic distribution was believed to comprise only the central and southern states of Brazil. We obtained partial sequences (about 500-570 bp) of first internal transcribed spacer (ITS1) nuclear ribosomal DNA from *Melipona* specimens putatively identified as *M. quinquefasciata* collected from different localities in northeastern Brazil. To confirm the taxonomic identity of the northeastern samples, specimens from the State of Goiás (Central region of Brazil) were included for comparison. All sequences were deposited in GenBank (accession numbers EU073751-EU073759). The mean nucleotide divergence (excluding sites with insertions/deletions) in the ITS1 sequences was only 1.4%, ranging from 0 to 4.1%. When the sites with insertions/deletions were also taken into account, sequence divergences varied from

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0 to 5.3%. In all pairwise comparisons, the ITS1 sequence from the specimens collected in Goiás was most divergent compared to the ITS1 sequences of the bees from the other locations. However, neighbor-joining phylogenetic analysis showed that all ITS1 sequences from northeastern specimens along with the sample of Goiás were resolved in a single clade with a bootstrap support of 100%. The ITS1 sequencing data thus support the occurrence of *M. quinquefasciata* in northeast Brazil.

Key words: *Melipona quinquefasciata*; Stingless bee; Nuclear ribosomal DNA; Internal transcribed spacer; Genetic variability

INTRODUCTION

Melipona quinquefasciata is a ground-nesting South American stingless bee species that was described by Lepeletier in 1836; it belongs to the subgenus *Melikerria* (Moure, 1975, 1992). Its known geographic distribution was believed to comprise only the southern states of Brazil, from the southern part of Espírito Santo to Rio Grande do Sul, including areas of Minas Gerais, Goiás, Mato Grosso, and Mato Grosso do Sul; it has also been reported from southern Bolivia, Paraguay and the north-northeast of Argentina (Mariano-Filho, 1911; Schwarz, 1932; Kerr, 1948; Moure, 1948, 1975; Viana and Melo, 1987). However, in 2002, Lima-Verde and Freitas (2002), based on taxonomic identification, reported the occurrence of *M. quinquefasciata* in two large plateaus of the State of Ceará, in northeast Brazil. Although traditional taxonomic classification, based on morphological and evolutionary traits, identified the bee population found in Ceará as *M. quinquefasciata*, some minor morphological variations of these individuals compared to voucher specimens from sites within the recognized occurrence area, along with the long distance (over 2000 km) from confirmed occurrence sites of this species, provoked doubts about its classification.

According to Lima-Verde and Freitas (2002), the presence of *M. quinquefasciata* in NE Brazil, so far from the other known populations of this species, could be explained by its isolation in remains of former vegetation communities nowadays found secluded in the caatinga phytocoenosis (tropical thorn scrub vegetation). This region was formerly part of the great Brazilian central plateau prior to its dismount as a consequence of climatic differentiation in northeastern Brazil, which began more intensively with the palaeoecological events that occurred in the Pliocene (Tertiary) and intensified during the Pleistocene (Quaternary).

Recent studies carried out with stingless bee species in Brazil have been able to separate into different species populations previously believed to belong to a single species, such as the case of *M. rufiventris* and *M. mondury* (Melo, 2003). However, morphological traits are not always sufficient for reliable distinction among species; fortunately, molecular genetics has appeared as a useful complimentary tool. The complete sequences of the first internal transcribed spacer (ITS1) of the nuclear ribosomal DNA from three *Melipona* species have been determined (Fernandes-Salomão et al., 2005). The relationships among eight species from this genus were inferred from partial ITS1 sequences, demonstrating the potential phylogenetic utility of this region (Fernandes-Salomão et al.,

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2005). More recently, the usefulness of this spacer for intraspecific studies in *Melipona* has also been evaluated in a study using *M. subnitida* specimens from different localities of northeastern Brazil (Cruz et al., 2006).

Population isolation is one of the recognized events leading to speciation (Silveira et al., 2002). We sequenced and compared a segment of the ITS1 from *M. quinquefasciata* collected from the States of Ceará, Piauí and Goiás in order to help determine whether the recently found bees in northeast Brazil are in fact a population of a discontinuous species, a sub-species or even a new species.

MATERIAL AND METHODS

Material collection

Adult specimens putatively identified as *M. quinquefasciata* were collected from various localities of the State of Ceará and from one locality in the State of Paiuí (Brazil). Specimens of *M. quinquefasciata* from Luziânia, State of Goiás, which is located in the Brazilian central plateau, were included for comparison. The bees were maintained in 100% ethanol until used for DNA extraction. Locality data, specimen voucher and GenBank accession numbers are listed in Table 1. Voucher specimens from all sampled localities were deposited in the bee collection of the Departamento de Zootecnia, Universidade Federal do Ceará, Fortaleza, CE, Brazil.

DNA purification

Total genomic DNA was isolated from five specimens of each locality using a CTAB-based protocol (Foster and Twell, 1996). The thorax of each specimen was ground in liquid nitrogen and digested for 2 h at 60°C in 500 µL CTAB extraction buffer (2% w/v CTAB, 100 mM Tris-HCl, pH 8.0, 20 mM EDTA, 1.4 M NaCl, 0.2% v/v 2-mercaptoethanol, 200 µg/mL proteinase K). DNA was then extracted sequentially with one volume of phenol: chloroform:isoamylalcohol (25:24:1) and one volume of chloroform:isoamylalcohol (24:1), and precipitated overnight at -20°C with two volumes of 100% ethanol. The precipitate was collected by centrifugation (8000 rpm, 20 min) and the DNA pellet was washed in 70% ethanol, air-dried, and resuspended in 100 µL 10 mM Tris-HCl, pH 8.0, 1 mM EDTA. The concentration of DNA in the various samples was determined by measuring the absorbance at 260 nm (A_{260}) of a 10-fold dilution of each sample. The quality of all DNA preparations was checked by 0.8% agarose gel electrophoresis, according to Sambrook et al. (1989).

Polymerase chain reaction amplification and DNA sequencing

Amplification reactions were performed in a final volume of 25 µL containing 500-800 ng genomic DNA (template), 20 mM Tris-HCl, pH 8.4, 50 mM KCl, 1.5 mM $MgCl_2$, 100 µM of each dATP, dCTP, dGTP, and dTTP (GE Healthcare, Piscataway, NJ, USA), 5 pmol of each primer and 0.5 units of Taq DNA Polymerase (GE Healthcare). Polymerase chain reaction (PCR) were carried out in an MJ-Research Inc. (Watertown, MD, USA) PTC-100 thermocycler programmed for an initial denaturation step (3 min at 94°C), followed by 45

cycles of 1 min at 94°C, 1 min at 55°C, and 2 min at 72°C. The last cycle was followed by a final incubation of 10 min at 72°C. The samples were then stored at 4°C until used. Amplified fragments were analyzed by standard horizontal electrophoresis on 1.0% agarose gels in TBE buffer (10 mM Tris-borate, 1 mM EDTA, pH 8.0) at 100 V. The DNA bands were stained with 0.5 µg/mL ethidium bromide (Sambrook et al., 1989). Control samples containing all reaction components except DNA were always tested to assure that no self-amplification or DNA contamination occurred. To obtain partial 3' end ITS1 sequences, the entire spacer was amplified using primers ITS2 (5'-GCTGCGTTCTTCATCGATGC-3') and ITS5 (5'-GCAAGTAAAAGTCGTAACAAGG-3'). The PCR products were diluted with distilled water and then sequenced in one direction using primer ITS2. Sequences were determined using the DYEnamic ET terminators cycle sequencing kit (GE Healthcare), following the protocol supplied by the manufacturer. Sequencing reactions were then analyzed in a MegaBACE 1000 automatic sequencer (GE Healthcare).

Sequence analysis

The quality of DNA sequences was checked, and overlapping fragments were assembled using the Phred/Phrap/Consed package (Ewing and Green, 1998; Ewing et al., 1998; Gordon et al., 1998). The BLAST program (Altschul et al., 1990) was employed to identify similarities between the isolated sequences and previously published data. Assembled high-quality (phred >20) sequences were aligned using CLUSTAL W (Thompson et al., 1994), with default gap penalties. Manual adjustments were made to improve the alignment, using the BioEdit software version 7.0.3 (Hall, 1999). A file containing the alignment is available upon request to the corresponding author. The multiple alignments were further analyzed using the computer packages MEGA3 (Molecular Evolutionary Genetics Analysis; Kumar et al., 2004) and DNASP (DNA Polymorphism Analysis version 4.10; Rozas et al., 2003). The evolutionary history was inferred using the neighbor-joining method (Saitou and Nei, 1987).

RESULTS AND DISCUSSION

Specimens of *M. quinquefasciata* from eight localities in northeastern Brazil were analyzed. Most of the samples (seven) were collected in the State of Ceará; one sample was from the State of Piauí (Table 1). Specimens from Luziânia, in the State of Goiás, were included as a representative sample from the recognized occurrence area for this species. Amplification of the ITS1 region alone using primers ITS2 and ITS5 and further analysis of the PCR products by 1% agarose gel electrophoresis revealed a single DNA band with ca. 1400 bp in all samples (data not shown). Fernandes-Salomão et al. (2005) reported ITS1 lengths (as estimated by agarose electrophoresis of PCR products) of 1430 bp (*M. quadrifasciata*, *M. mandacaia*, *M. favosa*, *M. bicolor*, *M. quinquefasciata*, and *M. compressipes*), 1540 bp (*M. scutellaris*, *M. capixaba* and *M. seminigra*), 1640 bp (*M. marginata*), and 1940 (*M. rufiventris*). Complete sequencing of ITS1 from *M. quadrifasciata*, *M. mandacaia* and *M. scutellaris* produced fragments of 1391, 1387, and 1417 bp, respectively (Fernandes-Salomão et al., 2005). Consequently, a significant size variation in the ITS1 exists in *Melipona*, and the ITS1 length in the genus is significantly greater than most published sequences, which are around 300 bp (e.g., from the tiger beetle *Cicindela dorsalis*; Vogler and DeSalle, 1994).

Table 1. Collection data of *Melipona quinquefasciata* specimens used for the partial sequencing of ITS1.

Sample name	Locality (county, state)	Coordinates	Altitude (m)	Voucher specimen	GenBank accession number
Araripe 1	Araripe, Ceará	07°19'55.8" S, 39°54'13.6" W	885	MGPA0604	EU073751
Araripe 3	Araripe, Ceará	07°19'24" S, 39°54'19" W	891	MGPA0804	EU073752
Flona 1	Crato, Ceará	07°09'30.6" S, 39°32'40.1" W	760	MGPA0803	EU073753
Flona 2	Crato, Ceará	07°09'35.2" S, 39°32'42.1" W	765	MGPA0903	EU073754
Flona 3	Crato, Ceará	07°09'33.4" S, 39°32'40.9" W	758	MGPA1003	EU073755
Jardim 1	Jardim, Ceará	07°29'44" S, 39°21'13.5" W	815	MGPA0204	EU073756
Piauí 1	Canto do Buriti, Piauí	08°06'36" S, 42°56'40" W	269	MGPA0305	EU073757
São Benedito	São Benedito, Ceará	04°02'55" S, 40°51'54" W	901	MGPA0205	EU073758
Goiás	Luziânia, Goiás	16°15'09" S, 47°57'01" W	930	MGPA0404	EU073759

Partial ITS1 sequences were obtained from the specimens of *M. quinquefasciata* and all the sequences were deposited in GenBank (accession numbers EU073751-EU073759). High-quality sequences corresponding to the 3' end of ITS1 varied from 491 bp (bees from Araripe, Ceará) to 572 bp (bees from Crato, Ceará). The G + C content ranged from 52.5 to 54.4%, with an average value of 54.3%. The multiple alignments of the *M. quinquefasciata* ITS1 fragments (excluding gap positions at both ends) had 499 sites, including 12 sites (2.4%) with gaps that were introduced during its optimization. Excluding the sites with insertions/deletions (indels), 462 sites (94.9%) were conserved, 25 sites (5.1%) were variable, 19 sites (3.9%) were unique to individual specimens, and six sites (1.2%) were informative for parsimony. Based on the trimmed multiple alignments, pairwise nucleotide comparisons (excluding indels) among all sequences revealed an average difference of about seven nucleotides, giving a mean divergence of only 1.4%. Nucleotide differences among aligned sequences (excluding indels) ranged from 0 (sequences from the samples Araripe1, Flona2 and Flona3 showed 100% identity to each other) to 20 (sequences from the samples Araripe3 and Goiás), thus giving a maximum divergence of about 4.1%. Nucleotide diversity Pi, i.e., the average number of nucleotide differences per site between two sequences, was 0.01409 ± 0.00481. When the sites with indels were considered, sequence divergence ranged from 0 (sequences from the samples Araripe1 and Flona2) to 5.3% (sequences from the samples Araripe3 and Góias), which corresponds to a sequence identity of 94.7%. Therefore, whether we excluded the sites with gaps or included them in the pairwise comparisons, the ITS1 sequence from the Goiás sample was most divergent from the rest of the sequences.

To clarify the relationships among the ITS1 sequences from *M. quinquefasciata*, a phylogenetic analysis was made, which also included the corresponding ITS1 sequences from other related species: *M. quadrifasciata*, *M. mandacaia*, *M. scutellaris* (Fernandes-Salomão et al., 2005) and *M. subnitida* (Cruz et al., 2006). The ITS1 sequences of *M. subnitida* were obtained from specimens collected from 14 localities across four States (Ceará, Maranhão, Paraíba, and Rio Grande do Norte) in northeastern Brazil, as described by Cruz et al. (2006). The multiple alignment of the *M. quinquefasciata* ITS1 sequences (from this study) with those of the other four species resulted in a data set with 25 sequences. This multiple alignment had 579 sites, with 144 gaps introduced manually to optimize alignment. Excluding the positions containing indels, 258 sites (59.3%) were conserved, 177 sites (40.7%) were variable, 108 sites (24.8%) were unique to individual specimens, and 69 sites (15.9%) were informative for parsimony. In the neighbor-joining tree (Figure 1), two major clades were obtained and supported by high

bootstrap values. One clade (97% bootstrap) comprised the ITS1 sequences from *M. subnitida*, *M. quadrifasciata*, and *M. mandacaia* (all belonging to subgenus *Melipona*), and another clade (100% bootstrap) contained the sequences from *M. quinquefasciata* (subgenus *Melikerria*).

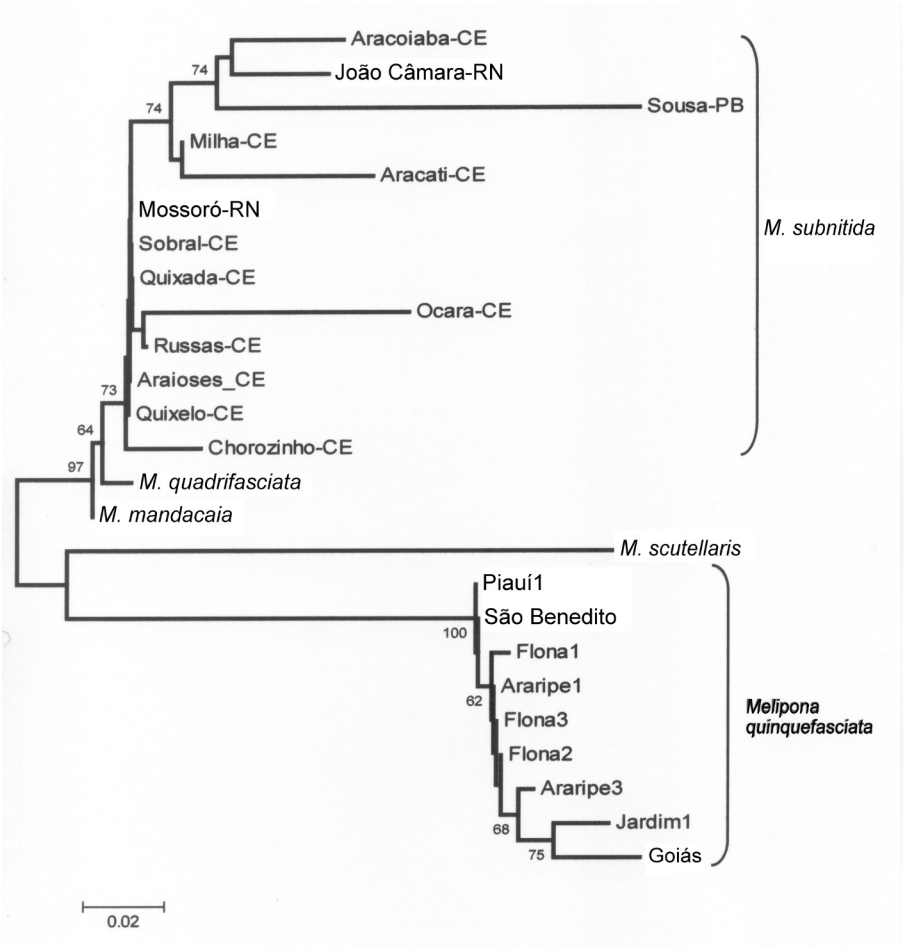

Figure 1. Neighbor-joining phylogenetic tree derived from partial ITS1 sequences of *Melipona quinquefasciata* specimens from northeastern Brazil and Goiás (GenBank accession numbers EU073751-EU073759). The sample names (for details, see Table 1) are shown at the terminal nodes. The ITS1 sequences from *M. subnitida* specimens collected from different localities (shown at terminal nodes) of the States of Maranhão (MA), Paraíba (PB), Rio Grande do Norte (RN), and Ceará (CE) were retrieved from GenBank (accession numbers DQ078726-DQ078738). Sequences of ITS1 from *M. quadrifasciata*, *M. mandacaia* and *M. scutellaris* were determined by Fernandes-Salomão et al. (2005). The optimal tree with the sum of branch lengths = 0.64852496 is shown. The percentages (only values above 50%) of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches (Felsenstein, 1985). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Tajima-Nei method (1984) and are in the units of the number of base substitutions per site. The rate variation among sites was modeled with a gamma distribution (shape parameter = 0.4337). All positions containing gaps were eliminated from the dataset (complete deletion option).

The ITS1 sequence from *M. scutellaris* (subgenus *Michmelia*) was resolved as a sister taxon of the clade containing the *M. quinquefasciata* data. Therefore, the recovery of a monophyletic clade containing the ITS1 sequence of *M. quinquefasciata* from Goiás and the other closely related sequences supported the finding that the ground-nesting *Melipona* bees occurring in NE Brazil, as previously reported by Lima-Verde and Freitas (2002), are indeed *M. quinquefasciata*.

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