

## Intraspecific variation in the first internal transcribed spacer (ITS1) of the nuclear ribosomal DNA in *Melipona subnitida* (Hymenoptera, Apidae), an endemic stingless bee from northeastern Brazil<sup>1</sup>

Darci de Oliveira CRUZ<sup>a</sup>, Daniel Macedo de Melo JORGE<sup>b</sup>, Júlio Otávio Portela PEREIRA<sup>a</sup>, Davi Coe TORRES<sup>b</sup>, Carlos Eduardo Alves SOARES<sup>b</sup>, Breno Magalhães FREITAS<sup>a</sup>, Thalles Barbosa GRANGEIRO<sup>b\*</sup>

<sup>a</sup> Grupo de Pesquisa com Abelhas, Departamento de Zootecnia, Centro de Ciências Agrárias, Universidade Federal do Ceará, Fortaleza-CE, Brazil

<sup>b</sup> Laboratório de Citogenética e Genética Molecular, Departamento de Biologia, Bloco 906, Centro de Ciências, Universidade Federal do Ceará, Av. Humberto Monte, s/n, CEP 60.455-970, Fortaleza-CE, Brazil

Received 27 June 2005 – revised 20 September 2005 – accepted 27 September 2005

**Abstract** – *Melipona subnitida* is endemic to northeastern Brazil where it has been exploited for the production of honey. In this work, partial sequences (about 600 bp) of the first internal transcribed spacer (ITS1) of the ribosomal DNA were obtained from *M. subnitida* specimens collected in thirteen localities in northeastern Brazil. All the sequences were deposited in GenBank (accession numbers DQ078726–DQ078738). The mean nucleotide divergence (excluding sites with insertions/deletions) in the ITS1 sequences was about 5%, ranging from 0 to 13%. However, when the sites with insertions/deletions were taken into account each sequence was unique, with nucleotide divergences varying from 1.1 to 18%. The intraspecific variation in the *M. subnitida* ITS1 is therefore greater than most of those previously published studies comprising a wide range of organisms. This high variation is taken as an evidence of isolated populations evolving individually for a long period of time. This information is also of importance for the development of appropriate conservation strategies for this species.

*Melipona subnitida* / stingless bee / nuclear ribosomal DNA / ITS/5.8S region / genetic variability

### 1. INTRODUCTION

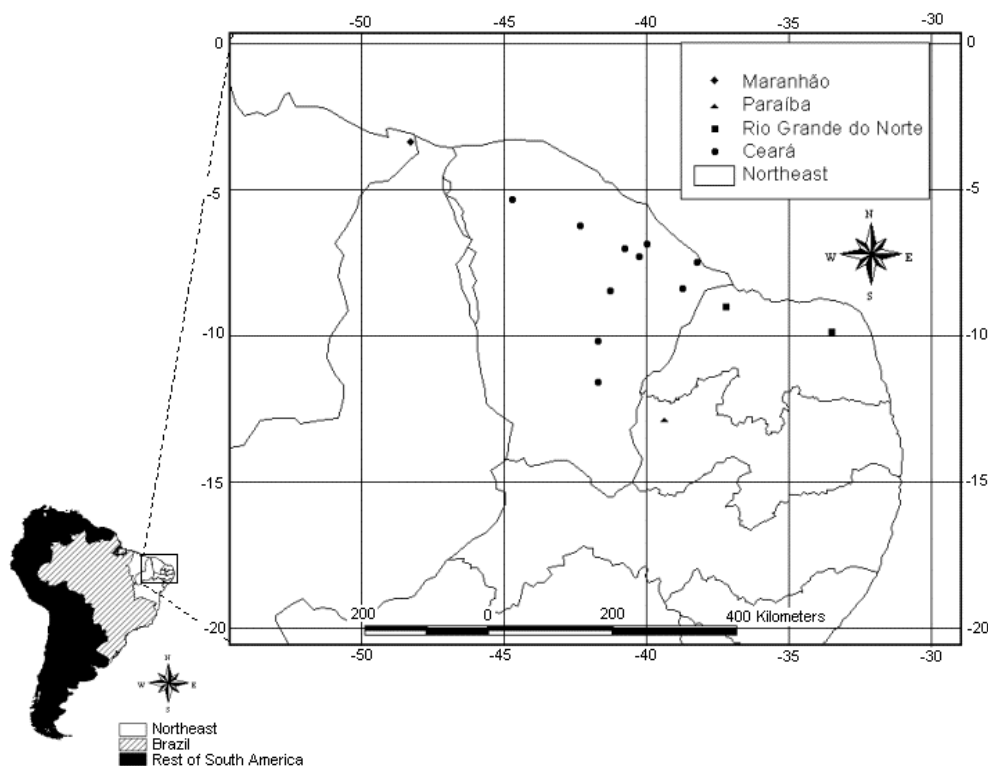
*Melipona* Illiger 1806 (Hymenoptera: Apidae) comprises about 40 neotropical species of stingless bees found exclusively in the equatorial, tropical and subtropical regions of the American continent, with its geographical distribution ranging from Mexico to Argentina (Schwarz, 1932; Michener and Sakagami, 1990; Camargo and Pedro, 1992; Michener, 2000). *Melipona subnitida* Ducke is endemic to northeastern Brazil where it is popularly known

as “jandaira”. This bee species, which makes its nests in the trunks of living trees, has been traditionally exploited for the production of honey (Bruening, 1990; Martins et al., 2004).

Despite their ecological relevance there are still a few molecular genetic studies of *Melipona* species (Fernandes-Salomão et al., 2002; Waldschmidt et al., 2002; Costa et al., 2005). More recently, the complete sequences of the first internal transcribed spacer (ITS1) of the nuclear ribosomal DNA (nrDNA) from three *Melipona* species were determined

\* Corresponding author: thalles@ufc.br

<sup>1</sup> Manuscript editor: Klaus Hartfelder



**Figure 1.** Map of the Northeast of Brazil showing sampling locations of *Melipona subnitida* specimens used in the present study. Specimens were collected in thirteen localities across the states of Maranhão (MA), Paraíba (PB), Rio Grande do Norte (RN) and Ceará (CE). The localities were: 1-Quixadá; 2-Sousa; 3-Ocara; 4-Mossoró; 5-Russas; 6-Sobral; 7-Milhã; 8-Quixelô; 9-Aracati; 10-Aracoiaiba; 11-João Câmara; 12-Chorozinho; and 13-Araioases.

(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., 2005). However, the usefulness of this spacer for intraspecific studies in *Melipona* has yet to be determined. In the present work the intraspecific sequence variation in the ITS1 was assessed in *M. subnitida* specimens from different localities of northeastern Brazil. Information on genetic variation is of great importance to understand the genetic structure as well as the phylogeographical patterns of a species. This information is also relevant for the development of effective conservation strategies.

## 2. MATERIALS AND METHODS

### 2.1. Insect material

Adult specimens of *M. subnitida* were collected in different localities of four states (Ceará, Paraíba, Rio Grande do Norte and Maranhão) in northeastern Brazil (Fig. 1). The Northeast of Brazil is situated between 1°02'–18°20' S and 34°47'–48°45' W, covering an area of 1 644 039 km<sup>2</sup>, from the state of Maranhão to the state of Bahia, which corresponds to 9.3% of the Brazilian territory (Andrade, 1977). Insects were maintained in 100% ethanol until used for DNA extraction. Locality data, specimen voucher and GenBank accession numbers are listed in Table I. Voucher specimens from all sampled localities are housed in the bee collection of the Departamento de Zootecnia, Universidade Federal do Ceará, Fortaleza-Ceará, Brazil.

**Table I.** Geographical and voucher data of the specimens of *Melipona subnitida* used in the present study.

Locality <sup>1</sup> (County-State)	Sample number <sup>2</sup>	Coordinates	Collection date	Voucher number	GenBank accession number
Quixadá-Ceará	1	4°58'17" S, 39°00'55" W	10/10/2003	MGPA0503	DQ078726
Sousa-Paraíba	2	6°45'33" S, 38°13'41" W	11/11/2003	MGPA0703	DQ078727
Ocara-Ceará	3	4°29'27" S, 38°35'48" W	25/08/2003	MGPA1305	DQ078728
Mossoró-Rio Grande do Norte	4	5°11'15" S, 37°20'39" W	05/04/2004	MGPA1404	DQ078729
Russas-Ceará	5	4°56'25" S, 37°58'33" W	30/10/2003	MGPA0603	DQ078730
Sobral-Ceará	6	3°41'10" S, 40°20'59" W	07/09/2003	MGPA0103	DQ078731
Milhã-Ceará	7	5°40'30" S, 39°11'38" W	21/09/2003	MGPA0104	DQ078732
Quixelô-Ceará	8	6°15'16" S, 39°12'07" W	21/09/2003	MGPA0203	DQ078733
Aracati-Ceará	9	4°33'42" S, 37°46'11" W	10/10/2003	MGPA0403	DQ078734
Aracoiaba-Ceará	10	4°22'16" S, 38°48'51" W	21/09/2003	MGPA0303	DQ078735
João Câmara-Rio Grande do Norte	11	5°32'15" S, 35°49'11" W	05/04/2004	MGPA1304	DQ078736
Chorozinho-Ceará	12	4°18'01" S, 38°29'52" W	28/09/2004	MGPA1104	DQ078737
Araioses-Maranhão	13	2°53'24" S, 41°54'11" W	06/07/2004	MGPA1704	DQ078738
General Sampaio-Ceará <sup>3</sup>	14	4°03'10" S, 39°27'16" W	06/04/2004	MGPA1204	-

<sup>1</sup> Specimens were collected in localities of northeastern Brazil; <sup>2</sup> As shown in Table II; <sup>3</sup> ITS1 sequence for specimens collected in this locality was not determined.

## 2.2. 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 1 volume of phenol:chloroform:isoamylalcohol (25:24:1) and 1 volume of chloroform:isoamylalcohol (24:1), and precipitated overnight at -20 °C with 2 volumes of 100% ethanol. The precipitate was collected by centrifugation (8 000 rpm, 20 min) and the DNA pellet was washed in 70% ethanol, air-dried, and resuspended in 100 µL of 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 ten-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).

## 2.3. PCR amplification and DNA sequencing

Amplification reactions were performed in a final volume of 25 µL containing 500–800 ng of genomic DNA (template), 20 mM Tris-HCl, pH 8.4, 50 mM KCl, 1.5 mM MgCl<sub>2</sub>, 100 µM of each dATP, dCTP, dGTP and dTTP (Amersham Biosciences, Sweden), 5 pmoles of each primer and 0.5 units of Taq DNA Polymerase (Amersham Biosciences, Sweden). PCR reactions were carried out in a MJ-Research Inc. (Watertown, Maryland, 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 as described before (Sambrook et al., 1989). Control samples containing all reaction components except

DNA were always used to test that no self-amplification or DNA contamination occurred. For the ITS/5.8S region, the primers used for amplification were ITS4 (TCCTCCGCTTATTGATATGC) and ITS5 (GCAAGTAAAAGTCGTAACAAGG), as suggested by Becerra and Venable (1999). These primers are complementary to the end of the 18S rDNA and to the beginning of the 28S rDNA, therefore they amplify a fragment of nrDNA containing ITS1, 5.8S rDNA and ITS2. To amplify only one of the two spacers, two additional primers, ITS2 (GCTGCGT-TCTTCATCGATGC) and ITS3 (GCATCGAT-GAAGAACGCAGC), were used (Becerra and Venable, 1999). To obtain partial 3' end ITS1 sequences, the spacer was amplified using primers ITS2 and ITS5, 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 sequencing kit (Amersham Biosciences Corp., USA) following the protocol supplied by the manufacturer. Sequencing reactions were then analysed in a MegaBACE 1000 automatic sequencer (Amersham Biosciences Corp., USA).

## 2.4. Sequence analysis

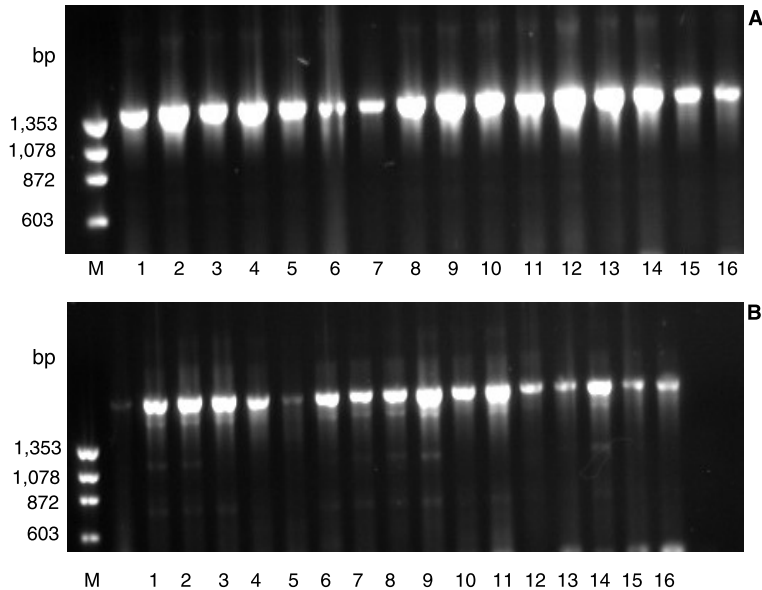
The quality of DNA sequences was checked and overlapping fragments were assembled using Phred/Phrap/Consed package (Ewing and Green, 1998; Ewing et al., 1998; Gordon et al., 1998). The program BLAST (Altschul et al., 1990) was employed to identify similarities between the isolated sequences and previously 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 software BioEdit 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).

## 3. RESULTS

In the present work, specimens of *M. subnitida* were analysed from fourteen localities across four states in northeastern Brazil (Fig. 1 and Tab. I). Specimens were from all known states (Ceará, Maranhão, Paraíba, and Rio Grande do Norte) where the species occurs, thus representing the entire range of its geographical distribution (Silveira et al., 2002).

The distances between the localities varied from ca. 23 km (between Chorozinho and Ocara) to about 737 km (between Araiões and João Câmara). Preliminary experiments using the primers ITS4 and ITS5 (Becerra and Venable, 1999), which amplify the whole ITS/5.8S region (ITS1 + 5.8S rDNA + ITS2) of the nrDNA, produced single DNA bands with a size in the range of 3650 to 3700 bp (data not shown) in all individuals of *M. subnitida* analyzed (Tab. I). Amplification of ITS1 region alone using primers ITS2 and ITS5 (Becerra and Venable, 1999) and further analysis of the PCR products by 1% agarose gel electrophoresis revealed DNA bands with ca. 1465 bp (Fig. 2A), with the estimated sizes ranging from 1445 bp to 1514 bp. ITS2 as amplified by primers ITS3 and ITS4 (Becerra and Venable, 1999) was larger than ITS1, and the mean size of spacer 2 band was about 2050 bp (Fig. 2B) ranging from 1995 bp (specimens from General Sampaio and Mossoró) to 2188 bp (specimens collected in Ocara).

Nucleotide sequencing was concentrated on the ITS1, as this region is more typically used in genetic analyses and also for the availability of ITS1 sequences from other *Melipona* species (Fernandes-Salomão et al., 2005). Therefore partial ITS1 sequences were obtained from the specimens of *M. subnitida* collected (Tab. I and Fig. 1) and all the sequences have been deposited in the GenBank (accession numbers DQ078726-DQ078738). High quality, assembled sequences corresponding to the 3' end of ITS1 varied from 589 bp (insects from Quixadá) to 610 bp (insects from Chorozinho). The G + C content ranged from 53 to 56.2%, with an average value of 54.8%. The multiple alignment of the ITS1 fragments had 654 sites, including 78 sites (11.9%) with gaps which were introduced during its optimization. Excluding the insertions/deletions (indels), a total of 437 sites (75.9%) were conserved, 139 sites (24.1%) were variable, 104 sites (18.1%) were unique to individual specimens and 35 sites (6.1%) were informative to parsimony. Pairwise nucleotide comparisons (excluding indels) among all sequences (data not shown) revealed an average difference of about 29 nucleotides, which represented a mean divergence of 5.2%. Nucleotide differences among aligned sequences (excluding indels) ranged from 0 to 75, thus representing a maximum



**Figure 2.** Agarose gel electrophoresis of PCR amplified ITS1 (A) and ITS2 (B) from specimens of *Melipona subnitida* used in this study. Localities and sample number are: Ocara-3 (lane 1), Sobral-6 (lane 2), Quixelô-8 (lane 3), Aracoiaba-10 (lane 4), Milha-7 (lane 5), Aracati-9 (lane 6), Quixadá-1 (lane 7), Russas-5 (lane 8), Chorozinho-12 (lane 9), General Sampaio-14 (lane 10), Sousa-2 (lane 11), Mossoró-4 (lane 12), João Câmara-11 (lane 13) and Araisos-13 (lanes 14–16).  $\phi$ X174 RF DNA digested with *Hae*III (250 ng) was used as a marker (lane M).

divergence of about 13%. Nucleotide diversity  $P_i$ , i.e., the average number of nucleotide differences per site between two sequences, was  $0.05086 \pm 0.01233$ . However, if the sites with gaps are considered, each sequence was unique with the highest pairwise similarity being 98.9% (1.1% sequence divergence) and the lowest similarity being 82% (18% divergence), as shown in Table II. A correlation between geographical distance and ITS1 sequence similarity could not be found as evidenced by clustering sequences using maximum parsimony analysis (Fig. 3). Although highly divergent, ITS1 sequences from all *M. subnitida* specimens clustered together with high support (bootstrap value 100%) as shown in Figure 3. Analysis based on the BLAST algorithm failed to reveal any nucleotide similarities between the isolated sequences and previously published sequences, except the ITS1 sequences from *M. quadrifasciata*, *M. mandacaia* and *M. scutellaris* (Fernandes-Salomão et al., 2005). To the best of our knowledge, the partial ITS1

sequences reported here are the first ones available in the literature from *M. subnitida*.

#### 4. DISCUSSION

The PCR amplified ITS1 and ITS2 regions of *M. subnitida* (this work), an endemic stingless bee found in northeastern Brazil, showed average lengths of 1465 and 2240 bp, respectively. For 16 *Melipona* species (not including *M. subnitida*), Fernandes-Salomão et al. (2002) 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 bp (*M. rufiventris*). Complete sequencing of ITS1 from *M. quadrifasciata*, *M. mandacaia* and *M. scutellaris* has produced fragments with 1391, 1387, and 1417 bp, respectively (Fernandes-Salomão

**Table II.** Pairwise nucleotide similarities (lower matrix) and divergences (upper matrix) between partial ITS1 sequences from *Melipona subnitida* collected in northeastern Brazil.

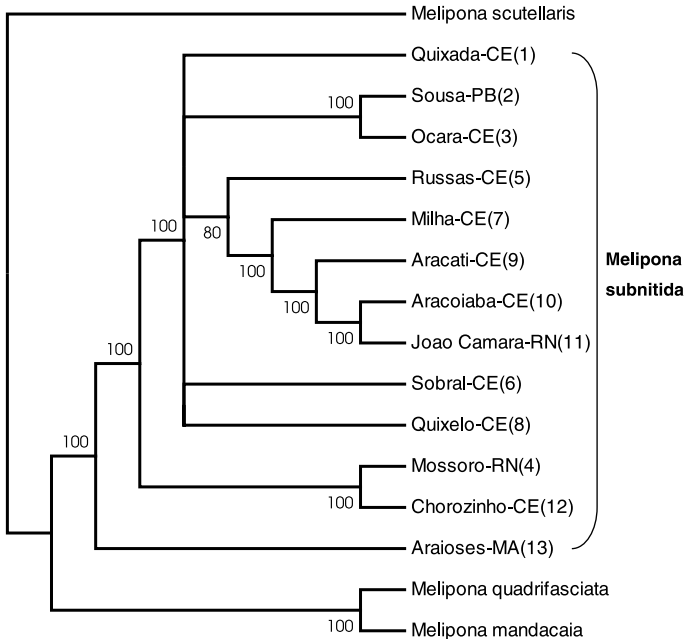
	1	2	3	4	5	6	7	8	9	10	11	12	13
1	-	0.127	0.097	0.021	0.016	0.012	0.017	0.011	0.080	0.101	0.072	0.071	0.012
2	0.873	-	0.180	0.124	0.133	0.131	0.131	0.131	0.169	0.174	0.154	0.176	0.128
3	0.903	0.820	-	0.105	0.099	0.094	0.099	0.097	0.146	0.157	0.134	0.150	0.094
4	0.979	0.876	0.895	-	0.022	0.019	0.027	0.022	0.080	0.106	0.081	0.079	0.020
5	0.984	0.867	0.901	0.978	-	0.014	0.017	0.017	0.080	0.106	0.077	0.071	0.016
6	0.988	0.869	0.906	0.981	0.986	-	0.017	0.011	0.077	0.097	0.071	0.072	0.012
7	0.983	0.869	0.901	0.973	0.983	0.983	-	0.017	0.072	0.096	0.067	0.072	0.016
8	0.989	0.869	0.903	0.978	0.983	0.989	0.983	-	0.077	0.099	0.077	0.066	0.012
9	0.920	0.831	0.854	0.920	0.920	0.923	0.928	0.923	-	0.116	0.118	0.129	0.073
10	0.899	0.826	0.843	0.894	0.894	0.903	0.904	0.901	0.884	-	0.099	0.152	0.099
11	0.928	0.846	0.866	0.919	0.923	0.927	0.933	0.923	0.882	0.901	-	0.123	0.075
12	0.929	0.824	0.850	0.921	0.929	0.928	0.928	0.934	0.871	0.848	0.877	-	0.071
13	0.988	0.872	0.906	0.981	0.984	0.988	0.984	0.988	0.927	0.901	0.925	0.929	-

Specimens were collected in the following localities: 1-Quixadá; 2-Sousa; 3-Ocara; 4-Mossoró; 5-Russas; 6-Sobral; 7-Milhã; 8-Quixelô; 9-Aracati; 10-Aracoiaba; 11-João Câmara; 12-Chorozinho; 13-Araioases; all sequences have been submitted to the GenBank (accession numbers DQ078726-DQ078738). All sites of aligned sequences were included in the comparisons.

et al., 2005). Therefore, a significant size variation in the ITS1 exists in the genus *Melipona*. In relation to ITS2, there are no previous reports about its size in other species of this genus. The ITS1 region of the nrDNA repeat in *Melipona* (this work and Fernandes-Salomão et al., 2002, 2005) and the ITS2 region of *M. subnitida* (present report) are much longer than most of those previously published. Typically, published ITS1 sequences are around 300 bp (e.g., the tiger beetle *C. dorsalis*; Vogler and DeSalle, 1994), while ITS2 sequences are typically 400–600 bp (e.g., the mosquito *Anopheles nuneztovari*; Fritz et al., 1994). In contrast, ITS1 lengths of three *Trichogramma* (Hymenoptera: Trichogrammatidae) species were found to be in the range 1300–1350 bp (Sappal et al., 1995). Previously, von der Schulenburg et al. (2001) had reported a unique

case of extreme length and length variation in the ITS1 elements of ladybird beetle (Coleoptera: Coccinellidae) species representing four subfamilies. In these ladybird beetles studied the ITS1 element ranged in length from 791 to 2572 bp (von der Schulenburg et al., 2001). Therefore, extreme size and size variation have evolved independently in different groups of insects.

ITS1 evolution seems to be shaped by internal repetition, leading to ITS1 size variation. This repetition includes repetitive elements with comparatively long repeat units or more commonly, simple repetitive sequence motifs (Vogler and DeSalle, 1994; Harris and Crandall, 2000; von der Schulenburg et al., 2001). In the partial ITS1 sequences of *M. subnitida* determined here, the presence of short, simple repetitions of one, two, three and four nucleotides



**Figure 3.** Consensus maximum parsimony tree derived from partial ITS1 sequences of *Melipona subnitida* specimens from northeastern Brazil (GenBank accession numbers DQ078726-DQ078738). Specimens were collected in thirteen localities across the states of Maranhão (MA), Paraíba (PB), Rio Grande do Norte (RN) e Ceará (CE). The localities sampled followed by sample number in parenthesis are shown at terminal nodes. Sequences from *M. quadrifasciata*, *M. mandacaia* and *M. scutellaris* were determined by Fernandes-Salomão et al. (2005) and used as outgroups. Bootstrap values (1 000 replicates) are shown at each node.

were found in all sampled individuals. Two major microsatellite loci with copy numbers  $\geq 3$  were identified:  $(GGA)_3$  and  $(AC)_8$ . In the tiger beetle (*Cicindela dorsalis*), Vogler and DeSalle (1994) showed the presence of two microsatellites in their ITS1 data set,  $(TA)_{5-9}$  and  $(GA)_{4-8}$ . In contrast, intraspecific variation in copy number within each microsatellite loci was not found in the ITS1 sequences from *M. subnitida* (present work).

The most striking result of the present work was the marked variation of the ITS1 sequences among *M. subnitida* specimens examined (Tab. II). Accordingly, the average ITS1 intraspecific variation in *M. subnitida* specimens, sampled in thirteen localities in northeastern Brazil, was about 5.2%, the sequence divergence ranging from 0 to 13%, when indels are not considered. Taking into account the sites with gaps, the range of sequence divergence was greater, from 1.1 to 18%. Therefore each sequence was unique when their patterns of indels are considered. Thus, there appears to be genetic structuring on at least a macrogeographic scale. The level of sequence divergence in the ITS1 region of *M. subnitida* is considerably greater than the values of most published studies on intraspecific ITS variation

for a variety of organisms (Chu et al., 2001; Luo et al., 2002; Otranto and Traversa, 2004). Intraspecific nucleotide divergence in ITS1 from *M. subnitida* is also greater than those found for ITS2 in different organisms. For example, intraspecific variation in *Aedes aegypti* ITS2 was only 1.17% (Wesson et al., 1992) while in *Anopheles sinensis* ITS2 divergence was even lower, 0.0–0.6% (Min et al., 2002). On the other hand, sequencing of ITS2 from specimens of *Anopheles rivulorum* collected from Eastern Africa (Kenya), Southern Africa (South Africa) and Western Africa (Burkina Faso) revealed that sequence divergence was 2% between specimens from South Africa and Kenya, and nearly tenfold higher (approximately 19%) between specimens from Burkina Faso and either South Africa or Kenya (Hackett et al., 2000). The authors took this high level of intraspecific ITS2 divergence as evidence that the Burkina Faso sample is not *An. rivulorum*, but rather a cryptic taxon within the Funestus Group (Hackett et al., 2000).

One explanation for the high divergence in ITS1 sequences of *M. subnitida* specimens could be a relatively ancient origin of this species. Indeed, stingless bees are some of the oldest known social bees. The oldest fossil bee is

a specimen of *Trigona prisca*, a stingless bee from the late Cretaceous New Jersey amber (96–74 million years before present), which is very similar to extant South American forest bees of the genus *Trigona* (Michener and Grimaldi, 1988). Other Meliponini taxa known from the fossil record include *Nogueirapis*, with a fossil species from the Miocene amber of Chiapas, Mexico (Wille, 1959, 1962) and *Proplebeia* from the Oligocene-Miocene amber of Dominican Republic (Michener, 1982). Although there is no known fossil *Melipona*, geological and biological data suggest that the genus was present at the end of the Cretaceous (Camargo et al., 1988; Camargo and Pedro, 1992).

Low rates of gene flow among populations may be another factor that has contributed for the observed high levels of genetic differentiation among *M. subnitida* specimens from geographically distinct places. This low rate of gene flow among populations would be explained by a limited capacity of dispersion (Hedrick, 1999). Accordingly, dispersion of Meliponini species occurs with the founding of new colonies through swarming. In this process, the dependence of the daughter nest from the mother colony for a certain period of time is a major factor that restricts the geographical distance between mother and daughter nests (Nogueira-Neto, 1954; Silveira et al., 2002). It also should be mentioned that, in contrast to *Apis mellifera* (Apini), whose colonies can swarm three to four times during a year, stingless bee colonies reproduce only once a year or even less frequently (Roubik, 1989). Environmental factors such as resource availability, suitable nesting site and predation play a role in colony multiplication (Roubik, 1989).

In the semi-arid region of northeastern Brazil, *M. subnitida* is a characteristic species of the bee fauna of the caatinga (Zanella, 2000), a xerophilous vegetation containing essentially spiny deciduous trees and shrubs in association with succulent plants, cacti and bromeliads (Kuhlmann, 1977). The climate is semi-arid with low annual rainfall and a dry-season of 7–9 months. Besides, there is a great variation in the annual precipitation and drought years and severe droughts are common (Andrade-Lima, 1981). Therefore, taking these factors into account, one could expect a significant variation in the frequency of swarming in *M. subnitida*,

which in turn would affect its geographical dispersal. In addition *M. subnitida* makes its nests in the trunks of living trees, and a fragmentation of its habitat due to increasing deforestation and agriculture intensification would further restrict the species dispersal (Martins et al., 2004).

No correlation was found between partial ITS1 sequence divergence among *M. subnitida* specimens and geographical distance of sampled localities as evidenced from the maximum parsimony tree (Fig. 3). At this stage, and assuming that most mutations in the ITS1 region are neutral (Schlötterer et al., 1994), one can conclude that sequence differences among *M. subnitida* specimens reflect isolated populations evolving individually for a long period of time. However, further analyses with wider sampling and the use of other markers are needed for a comprehensive understanding of the population genetics and phylogeography of the species. The relatively high genetic intraspecific variation found in *M. subnitida* as assessed by partial sequencing of ITS1 also suggest that preservation of as many populations as possible would be important for conservation purposes.

## ACKNOWLEDGMENTS

This work was supported by grants from the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Fundação Cearense de Apoio ao Desenvolvimento Científico e Tecnológico (FUNCAP), and Banco do Nordeste do Brasil (BNB). T.B. Grangeiro and B.M. Freitas have research fellowships from CNPq.

**Résumé – Variation intraspécifique du premier espaceur transcrit interne (ITS1) de l'ADN ribosomal nucléaire chez *Melipona subnitida* (Hymenoptera, Apidae), abeille sans aiguillon endémique du nord-est du Brésil.** *Melipona subnitida* Ducke est une abeille sans aiguillon endémique du nord-est du Brésil. Malgré son importance économique dans la production locale de miel, la variabilité génétique de cette abeille n'a pas encore été étudiée. Dans ce but nous avons séquencé une partie du premier espaceur transcrit interne (ITS1) de l'ADN ribosomal. Pour estimer la variabilité intraspécifique nous avons prélevé des échantillons d'abeilles dans diverses localités du nord-est du Brésil (Tab. I et Fig. 1). L'électrophorèse sur gel d'agarose a permis d'évaluer les tailles de l'ITS1 et l'ITS2 à 1465 et 2240 paires de bases



respectivement (Fig. 2). Les séquences partielles de l'ITS1 (environ 600 nucléotides) des échantillons de ces localités ont été déposées dans la GenBank (numéros d'accès DQ078726-DQ078738). La répétition de séquences courtes et simples de un, deux, trois et quatre nucléotides ont été trouvées dans toutes les abeilles échantillonnées. Nous avons aussi identifié deux locus importants de microsatellites avec des nombres de copies de 3 répétitions ou plus, (GGA)<sub>3</sub> et (AC)<sub>8</sub>, mais ils n'ont présenté aucune variabilité intraspécifique. La divergence moyenne des nucléotides (non compris les sites d'insertions/délétions dans les alignements) entre les séquences partielles d'ITS1 était comprise entre 0 et 13 %, avec une valeur moyenne de 5 %. Mais lorsqu'on a pris en compte les sites d'insertions/délétions, chaque séquence était unique avec une divergence des nucléotides entre 1,1 et 18 % (Tab. II). La variation intraspécifique de l'ITS1 de *M. subnitida* est donc plus grande que les valeurs publiées jusqu'à ce jour pour d'autres organismes. Nous n'avons trouvé en outre aucune corrélation entre la divergence des séquences et la distance géographique des échantillons (Fig. 3). Nous considérons la forte variabilité intraspécifique de l'ITS1 de *M. subnitida* comme la preuve que des populations isolées ont évolué individuellement sur une longue période. Notre étude montre le fort potentiel de la région de l'ITS1 comme outil moléculaire pour étudier la génétique des populations et la phylogéographie des espèces. Ces informations sont importantes pour mettre au point des stratégies appropriées de conservation.

***Melipona subnitida* / abeille sans aiguillon / ADN ribosomal nucléaire / région ITS/5,8 / variabilité génétique**

**Zusammenfassung – Intraspezifische Variation im ersten Internen Transkribierten Spacer (ITS1) der nukleären ribosomalen DNA von *Melipona subnitida* (Hymenoptera, Apidae), einer endemischen stachellosen Biene aus dem Nordosten Brasiliens.** *Melipona subnitida* Ducke ist eine endemische stachellose Biene aus dem Nordosten von Brasilien. Trotz ihrer ökonomischen Bedeutung in der lokalen Honigproduktion ist diese Biene vor allem im Hinblick auf ihre genetische Variabilität noch wenig untersucht. Zu diesem Zweck sequenzierten wir einen Teil des ersten Internen Transkribierten Spacers (ITS1) der ribosomalen DNA. Für die Erfassung der intraspezifischen Variabilität wurden Bienenproben von verschiedenen Orten im Nordosten Brasiliens untersucht (Tab. I und Abb. 1). Mittels Agarosegelelektrophorese wurden die mittleren Molekülgrößen von *M. subnitida* ITS1 und ITS2 auf 1 465, bzw. 2240 Basenpaare geschätzt. Die partiellen ITS1-Sequenzen (von jeweils ungefähr 600 Nukleotiden) von Bienenproben dieser Lokalitäten wurden in GenBank deponiert (Zugriffsnummern DQ078726-DQ078738). Wir fanden kurze, einfache Sequenzwiederholungen von

eins, zwei, drei oder vier Nukleotiden in den DNA-Proben aller gesammelten Bienen. Ausserdem identifizierten wir zwei grössere Mikrosatelliten-Loci mit Kopienzahlen von drei und mehr Wiederholungen, (GGA)<sub>3</sub> und (AC)<sub>8</sub>, die allerdings keine intraspezifische Variabilität zeigten. Die mittlere Nukleotid-Divergenz (ohne Berücksichtigung von Insertionen/Deletionen in den Sequenzvergleichen) zwischen den partiellen ITS1-Sequenzen betrug 5 %, mit einer Variationsbreite von 0–13 %. Bei Einbeziehung der Positionen mit Insertionen/Deletionen wurden alle Sequenzen als singular klassifiziert, mit einer Nukleotid-Divergenz zwischen 1,1 und 18 % (Tab. II). Die intraspezifische Variabilität in den *M. subnitida* ITS1-Sequenzen ist demzufolge grösser als die bisher für andere Organismen publizierten Werte. Des weiteren fanden wir keine Korrelation zwischen Sequenzdivergenz und geographischer Distanz der Bienenproben (Abb. 3). Wir vermuten, dass die hohe intraspezifische ITS1-Variabilität von *M. subnitida* auf eine lange zeitliche Trennung isolierter Populationen zurückzuführen ist. Die gegenwärtige Studie zeigt ausserdem das hohe Potential der ITS1-Region in molekularen Analysen zur Populationsgenetik und Phylogeographie, und derartige Informationen sind insbesondere auch für die Entwicklung von Konservierungsstrategien einzelner Spezies von Bedeutung.

***Melipona subnitida* / stachellose Bienen / nukleäre ribosomale DNA / ITS/5,8S Region / genetische Variabilität**

**REFERENCES**

- Andrade G.O. (1977) Alguns aspectos do quadro natural do Nordeste, Sudene, Recife.
- Andrade-Lima D. (1981) The caatingas dominium, Rev. Bras. Bot. 4, 149–153.
- Altschul S.F., Gish W., Miller W., Myers E.W., Lipman D.J. (1990) Basic local alignment search tool, J. Mol. Biol. 215, 403–410.
- Becerra J.X., Venable D.L. (1999) Nuclear Ribosomal DNA Phylogeny and ITS Implications for evolutionary trends in Mexican *Burserea* (Bursereaceae), Am. J. Bot. 86, 1047–1057.
- Bruening P.H. (1990) Abelha Jandaira, ESAM, Coleção Mossoroense C 557, Mossoró.
- Camargo J.M.F., Pedro S.R.M. (1992) Systematics, phylogeny and biogeography of Meliponinae (Hymenoptera, Apidae), a mini-review, Apidologie 23, 509–522.
- Camargo J.M.F., Moure J.S., Roubik D.W. (1988) *Melipona yucatanica*, a new species (Hymenoptera: Apidae: Meliponinae): stingless bee dispersal across the caribbean arc and post-eocene variation, Pan-Pacific Entomol. 64, 147–157.
- Costa R.G., Tavares M.G., Dias L.A., Campos L.A. (2005) Isoenzyme variation in *Melipona rufiventris* (Hymenoptera: Apidae, Meliponina)

- in Minas Gerais State, Brazil, *Biochem. Genet.* 43, 49–58.
- Chu K.H., Li C.P., Ho H.Y. (2001) The first internal transcribed spacer (ITS-1) of ribosomal DNA as a molecular marker for phylogenetic and population analyses in crustacean, *Mar. Biotechnol.* (NY) 3, 355–361.
- Ewing B., Green P. (1998) Base-Calling of Automated Sequencer Traces Using Phred. II. Error Probabilities, *Genome Res.* 8, 186–194.
- Ewing B., Hillier L., Wendl M.C., Green P. (1998) Base-Calling of Automated Sequencer Traces Using Phred. I. Accuracy Assessment, *Genome Res.* 8, 175–185.
- Fernandes-Salomão T.M., Muro-Abad J.I., Campos L.A.O., Araújo E.F. (2002) Mitochondrial and nuclear DNA characterization in the *Melipona* species (Hymenoptera, Meliponini) by RFLP analysis, *Hereditas* 137, 229–233.
- Fernandes-Salomão T.M., Rocha R.B., Campos L.A.O., Araújo E.F. (2005) The first internal transcribed spacer (ITS-1) of *Melipona* species (Hymenoptera, Apidae, Meliponini): characterization and phylogenetic analysis, *Insectes Soc.* 52, 11–18.
- Foster G.D., Twell D. (1996) *Plant gene isolation, Principles and practice*, John Wiley & Sons, England.
- Fritz G.N., Conn J., Cockburn A., Seawright J. (1994) Sequence analysis of the ribosomal DNA internal transcribed spacer 2 from populations of *Anopheles nuneztovari* (Diptera: Culicidae), *Mol. Biol. Evol.* 11, 406–416.
- Gordon D., Abajian C., Green P. (1998) Consed: A Graphical Tool for Sequence Finishing, *Genome Res.* 8, 195–202.
- Hackett B.J., Gimmig J., Guelbeogo W., Costantini C., Koekemoer L.L., Coetzee M., Collins F.H., Besansky N.J. (2000) Ribosomal DNA internal transcribed spacer (ITS2) sequences differentiate *Anopheles funestus* and *An. rivulorum*, and uncover a cryptic taxon, *Insect Mol. Biol.* 9, 369–374.
- Hall T.A. (1999) BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT, *Nucleic Acids Symp. Ser.* 41, 95–98.
- Harris D.J., Crandall K.A. (2000) Intragenomic variation within ITS1 and ITS2 of freshwater crayfishes (Decapoda: Cambaridae): implications for phylogenetic and microsatellite studies, *Mol. Biol. Evol.* 17, 284–291.
- Hedrick P.W. (1999) *Genetics of populations*, Jones and Bartlett Publishers, Massachusetts.
- Kuhlmann E. (1977) A vegetação, in: *Geografia do Brasil-Região Nordeste* Vol. 2, IBGE, Rio de Janeiro, pp. 85–110.
- Kumar S., Tamura K., Nei M. (2004) MEGA3: Integrated software for Molecular Evolutionary Genetics Analysis and sequence alignment, *Brief. Bioinform.* 5, 150–163.
- Luo H.Y., Nie P., Zhang Y.A., Wang G.T., Yao W.J. (2002) Molecular variation of *Bothriocephalus acheilognathi* Yamaguti, 1934 (Cestoda: Pseudophyllidea) in different fish host species based on ITS rDNA sequences, *Syst. Parasitol.* 52, 159–166.
- Martins C.F., Cortopassi-Laurino M., Koedam D., Imperatriz-Fonseca V.L. (2004) Tree species used for nidification by stingless bees in the Brazilian caatinga (Seridó, PB; João Câmara, RN), *Biota Neotropica* 4, available on line at: <http://www.biotaneotropica.org.br/v4n2/en/abstract?article+BN00104022004> (accessed on 14 December 2005).
- Michener C.D. (1982) A new interpretation of fossil social bees from the Dominican Republic, *Sociobiology* 7, 37–45.
- Michener C.D. (2000) *The bees of the world*, The John Hopkins University Press, Baltimore and London.
- Michener C.D., Grimaldi D.A. (1988) The oldest fossil bee: Apoid history, evolutionary stasis, and antiquity of social behavior, *Proc. Natl. Acad. Sci. (USA)* 85, 6424–6426.
- Michener C.D., Sakagami S.F. (1990) Classification of the Apidae (Hymenoptera), *Univ. Kansas Sci. Bull.* 54, 75–164.
- Min G.S., Choochote W., Jitpakdi A., Kim S.J., Kim W., Jung J., Junkum A. (2002) Intraspecific hybridization of *Anopheles sinensis* (Diptera: Culicidae) strains from Thailand and Korea, *Mol. Cells* 14, 198–204.
- Nogueira-Neto P. (1954) Notas bionômicas sobre Meliponíneos III – Sobre a enxameagem (Hym. Apoidea), *Arq. Mus. Nac. (Rio)* 42, 419–452.
- Otranto D., Traversa D. (2004) Molecular characterization of the first internal transcribed spacer of ribosomal DNA of the most common species of eyeworms (Thelazioidea: Thelazia), *J. Parasitol.* 90, 185–188.
- Roubik D.W. (1989) *Ecology and natural history of tropical bees*, Cambridge University Press, New York.
- Rozas J., Sanchez-DelBarrio J.C., Messeguer X., Rozas R. (2003) DnaSP, DNA polymorphism analyses by the coalescent and other methods, *Bioinformatics* 19, 2496–2497.
- Sambrook J., Fritsch E.F., Maniatis T. (1989) *Molecular cloning: a laboratory manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.
- Sappal N.P., Jeng R.S., Hubbes M., Liu F. (1995) Restriction fragment length polymorphisms in polymerase chain reaction amplified ribosomal DNAs of three *Trichogramma* (Hymenoptera: Trichogrammatidae) species, *Genome* 38, 419–425.
- Schlötterer C., Hauser M.-T., von Haeseler A., Tautz D. (1994) Comparative evolutionary analysis of rDNA ITS regions in *Drosophila*, *Mol. Biol. Evol.* 11, 513–522.
- Schwarz H.F. (1932) The genus *Melipona*, The type genus of Meliponidae or stingless bees, *Bull. Am. Mus. Nat. Hist.* 63, 231–459.

- Silveira F.A., Melo G.A.R., Almeida E.A.B. (2002) Abelhas brasileiras: sistemática e identificação, Belo Horizonte.
- Thompson J.D., Higgins D.G., Gibson T.J. (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice, *Nucleic Acids Res.* 22, 4673–4680.
- Vogler A.P., DeSalle R. (1994) Evolution and phylogenetic information content of the ITS-1 region in the tiger beetle, *Cicindela dorsalis*, *Mol. Biol. Evol.* 11, 393–405.
- von der Schulenburg J.H.G., Hancock J.M., Pagnamenta A., Sloggett J.J., Majerus M.E.N., Hurst G.D.D. (2001) Extreme length and length variation in the first ribosomal internal transcribed spacer of ladybird beetles (Coleoptera: Coccinellidae), *Mol. Biol. Evol.* 18, 648–660.
- Waldschmidt A.M., Marco-Junior P., Barros E.G., Campos L.A. (2002) Genetic analysis of *Melipona quadrifasciata* LEP. (Hymenoptera: Apidae, Meliponinae) with RAPD markers, *Braz. J. Biol.* 62, 923–928.
- Wesson D.M., Porter C.H., Collins F.H. (1992) Sequence and secondary structure comparisons of ITS rDNA in mosquitoes (Diptera: Culicidae), *Mol. Phylogenet. Evol.* 1, 253–269.
- Wille A. (1959) A new fossil stingless bee (Meliponini) from the amber of Chiapas, Mexico, *J. Paleontol.* 33, 849–852.
- Wille A. (1962) A revision of the subgenus *Nogueirapis*; an archaic group of stingless bees (Hymenoptera: Apidae), *J. New York Entomol. S.* 70, 218–234.
- Zanella F.C.V. (2000) The bees of the Caatinga (Hymenoptera, Apoidea, Apiformes): a species list and comparative notes regarding their distribution, *Apidologie* 31, 579–592.