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Original article

Mitochondrial DNA restriction and genomic maps of seven species of *Melipona* (Apidae: Meliponini)

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Abstract – This paper reports efforts to characterize the mitochondrial genome of Meliponini. Here we describe the restriction and partial genomic map of seven *Melipona* species (*M. bicolor*, *M. compressipes*, *M. marginata*, *M. melanoventer*, *M. quadrifasciata*, *M. rufiventris* and *M. subnitida*). The maps were obtained through RFLP and PCR-RFLP using 15 restriction enzymes. The total number of sites mapped ranged from 12 to 19, indicating a high level of genetic diversity among those species. MtDNA total size was estimated to be 18 500 bp.

***Melipona* / stingless bee / mtDNA / rflp / restriction map**

1. INTRODUCTION

In a recent bee systematic review, Michener (2000) divided the family Apidae in three subfamilies: Xylocopinae, Nomadinae and Apinae. The actual subfamily Apinae comprises 19 tribes including Apini, Meliponini, Bombini and Euglossini, the "corbiculate" bees. Those 4 tribes used to define the family Apidae before Michener's revision. Although Apini is the most studied tribe of the family, it presents only one genus (*Apis* L.) and about 11 valid species naturally distributed in the Old World (Michener, 2000). Conversely, Meliponini is the most diverse group, presenting several hundred species distributed in tropical and southern subtropical areas of the World. The total number of species is still not established mainly because of the high number of cryptic species (Michener, 2000). Some aspects of the phylogeny and classification of Meliponini were discussed by Camargo and Pedro (1992) and Michener (2000).

Most of the studies conducted on Meliponini have been based on morphology and behavior. However, recently, studies have been published on molecular markers suitable to investigate aspects of the biology and evolution of this group of bees (Peters et al., 1998; Bezerra, 1999; Paxton et al., 1999; Francisco et al., 2001; Costa et al., 2003). In general, mitochondrial DNA (mtDNA) analyses have proved useful due to characteristics that include maternal inheritance, high level of nucleotide substitution and small molecular size (16 kb in average). Analysis of mtDNA polymorphism has been applied to solve questions concerning population dynamics, species and subspecies characterization and phylogeny. Within the bees, mtDNA analysis has been largely employed but almost exclusively to the genus *Apis* (Arias et al., 1990; Sheppard et al., 1991; Garnery et al., 1992; Lobo, 1995; Arias and Sheppard, 1996; Arias et al., 1996; Franck et al., 2000; Meixner et al., 2000; Smith et al., 2000).

In a previous paper we described the mtDNA characterization of five Meliponini species belonging to the genus *Plebeia* Schwarz (Francisco et al., 2001). The restriction maps

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are now being used as a guide to precisely locate polymorphic restriction sites in population surveys and make inferences about population dynamics and phylogeography.

In this paper we report the restriction and partial genomic map of seven species of *Melipona* Illiger. This genus presents a neotropical distribution, and seems to be the most derivative group, suggesting a post-Gondwanan origin (Camargo and Pedro, 1992). The number of *Melipona* species is estimated to be around 40, geographically distributed from Mexico to Argentina (Michener, 2000). In Brazil some species are suffering population constraints due to forest devastation (Kerr, unpublished data) and probably some species are facing extinction. The genus *Melipona* plays a very important role as pollinators and also some species are domesticated for honey harvesting.

Knowing the potential of mtDNA markers through *Apis* and *Plebeia* examples, the characterization of mitochondrial genome of *Melipona* species may contribute further to conduct population surveys in an attempt to understand the intra and interspecific genetic variability, biogeography, population dynamics and also evolution of the genus.

2. MATERIALS AND METHODS

The species and subspecies studied in this present work were the following: *Melipona bicolor* (Lepeletier), *M. compressipes* (Fabricius), *M. marginata* (Lepeletier), *M. melanoventer* (Schwarz), *M. quadrifasciata quadrifasciata* (Lepeletier), *M. quadrifasciata anthidioides* (Lepeletier), *M. rufiventris* (Lepeletier), and *M. subnitida* (Ducke). One nest per species or subspecies was sampled. Individuals of *M. bicolor*, *M. marginata*, *M. quadrifasciata quadrifasciata*, *M. quadrifasciata anthidioides*, and *M. subnitida* were collected from hives maintained at the Laboratório de Abelhas do Departamento de Ecologia do IB-USP. The colony of *M. subnitida* was originally from the northeast region of Brazil while the others were originally from Cunha, SP. The samples of *M. compressipes*, *M. melanoventer* and *M. rufiventris* were brought in alcohol from Pará, Brazil.

2.1. DNA extraction

Total DNA was extracted according to the protocol TNE described by Sheppard and McPheron (1991) and used in Restriction Fragment Length Polymorphism (RFLP) experiments. For mtDNA genomic amplification through Polymerase Chain Reaction (PCR) the DNA extractions were performed following the procedure modified by Arias and Sheppard (1996). One or two thoraces were used per extraction. This number varied depending on the bee size, e.g. for *M. bicolor* DNA extractions one thorax was used and for *M. marginata* two thoraces.

2.2. RFLP and Southern blot analysis

The DNA samples were screened using the following restriction enzymes: *Bam*H I, *Bcl* I, *Cfo* I, *Cla* I, *Eco*R V, *Hae* III, *Hpa* I, *Pvu* II and *Sca* I (Boehringer Mannheim Biochemica), *Bgl* II, *Eco*R I, *Hin*d III, *Pst* I, *Xba* I and *Xho* I (GIBCO BRL). The digestions were performed using 10% of the total volume of TNE extractions and 5 U of enzyme, following the manufactures instructions, and incubated overnight. For double digestions, the DNA was first digested overnight with one enzyme, precipitated, and then digested with the second enzyme.

The RFLP products were analyzed by electrophoresis in 0.8% agarose gels, stained with ethidium bromide and visualized and photographed under UV light. The DNA fragments were transferred to nylon membrane (Amersham Pharmacia) and hybridized with heterospecific probe at 56 °C overnight. The mtDNA probe was obtained from *Apis mellifera* through PCR amplifications (Francisco et al., 2001). The DIG DNA Labeling and Detection Kit (Roche) was used in the next steps following the manufacturer's protocol.

2.3. Polymerase Chain Reactions (PCR)

Amplifications of ten mtDNA regions were performed using primers and PCR conditions already published (Francisco et al., 2001). Some modifications on the annealing temperature were necessary for the pairs of primers 3, 7 and 9 (Tab. I in Francisco et al., 2001), here we used 43 °C, 43 °C, and 42 °C, respectively. The reactions were performed in 50 μL, containing 1 μL of DNA, 5 μL of 10X PCR buffer, 1.5 μL of each primer (20 mM), 5 μL of dNTPs (2 mM each), 2.5 U of *Taq* DNA polymerase (Boehringer Mannheim Biochemica) and sterile water. The PCR products were analyzed in 0.8% agarose gels, stained with ethidium bromide, visualized and photographed under UV light.

For the PCR-RFLP technique, the PCR products were digested (single digestion) with the same restriction enzymes used for the total DNA. The fragments generated were separated in 1.5% Nusieve (FMC) 3:1 agarose gels.

Table I. Number of restriction sites generated per enzyme for each species. In parenthesis are the corresponding abbreviations used in Figure 1.

Enzymes	*M. bicolor*	*M. compressipes*	*M. marginata*	*M. melanoventer*	*M. q. anthidioides*	*M. q. quadrifasciata*	*M. rufiventris*	*M. subnitida*
*Bam*H I	0	0	0	0	0	0	0	0
Bcl I (B)	3	3	3	3	3	3	3	3
Bgl II (G)	1	1	1	1	0	1	1	0
Cfo I	0	0	0	0	0	0	0	0
Cla I (C)	3	3	1	1	3	3	1	3
*Eco*R I (E)	2	2	3	1	3	3	1	2
*Eco*R V (V)	1	0	2	0	0	0	0	1
Hae III (A)	1	1	1	0	1	1	0	1
*Hin*d III (D)	4	3	3	2	3	3	3	3
Hpa I	0	0	0	0	0	0	0	0
Pst I (P)	2	1	3	2	2	2	1	2
Pvu II (U)	0	0	1	0	0	0	0	0
Sca I (S)	0	0	0	0	1	1	0	1
Xba I (X)	1	1	1	2	1	1	2	1
Xho I	0	0	0	0	0	0	0	0
Total	18	15	19	12	17	18	12	17

3. RESULTS AND DISCUSSION

A total of 27 restriction sites were generated by 11 restriction enzymes among the 7 *Melipona* species studied (Tab. I). The enzymes *Bam*H I, *Cfo* I, *Hpa* I, and *Xho* I did not cut the mtDNA of any species. The total number of restriction sites per species varied, being the lowest verified in *M. rufiventris* and *M. melanoventer* (12 sites) and the highest in *M. marginata* (19 sites).

The Southern blot data enabled us to determine fragment length by comparison with molecular weight markers and, consequently the mtDNA total size could be estimated. The entire molecule size is approximately 18 500 bp, and differences among the species were not verified by the methodology applied. The same mitochondrial genome size had been previously reported for *Plebeia* species (Francisco et al., 2001). In comparison to *A. mellifera*, *Melipona* and *Plebeia* mtDNA are about 2200 bp longer. However, some mitochondrial regions amplified through PCR presented smaller sizes than expected comparing to the corresponding regions in *A. mellifera*. The total difference for these defined regions was a decrease of around 300 bp compared to *Apis*, although the total genome is still 2200 bp larger. The same fact, and for the same PCR amplified regions, was reported for *Plebeia* mtDNA (Francisco et al., 2001). These present data corroborate our previous hypothesis that absence of intergenic regions (especially the major one located between the genes COI and COII), tRNA transpositions, and large insertions in the A+T rich region are responsible for the mtDNA size differences verified between the tribes Meliponini and Apini (Silvestre et al., 2001; Silvestre et al., 2002). Those events (insertions, deletions, and translocations) have been described in the literature as responsible for genome size differences even among species of the same genus (Fauron and Wolstenholme, 1976; Rawlings et al., 2001).

Restriction maps (Fig. 1) were built for each species based on data from Southern blot and PCR-RFLP. The analysis of the aforementioned maps revealed seven conserved sites among the species and eight sites species specific by their presence or absence. Five out of these eight sites are related to *M. marginata*.

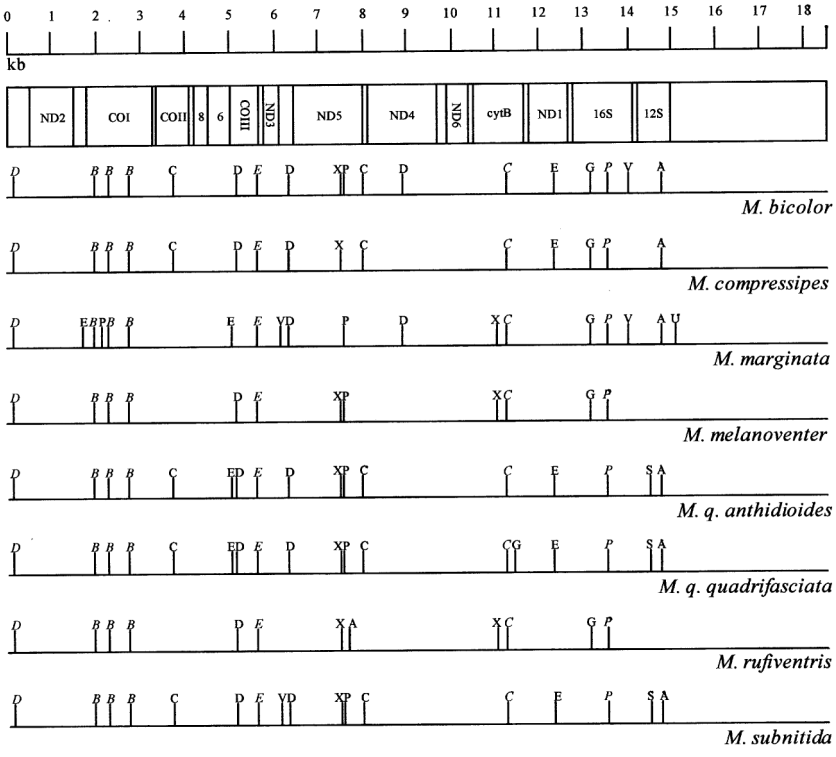

Figure 1. Linear mtDNA restriction site maps and relative main gene positions for *Melipona* species, using *Apis mellifera* mitochondrial DNA gene order as a guide (Crozier and Crozier, 1993). The italicized letters refer to conserved sites. B, *Bcl* I; G, *Bgl* II; C, *Cla* I; E, *Eco*R I; V, *Eco*R V; A, *Hae* III; D, *Hin*d III; P, *Pst* I; S, *Sca* I; X, *Xba* I. ND2, NADH dehydrogenase subunit 2; COI, cytochrome c oxidase subunit 1; COII, cytochrome c oxidase subunit 2; 8, ATP F_0 synthase subunit 8; 6, ATP F_0 synthase subunit 6; COIII, cytochrome c oxidase subunit 3; ND3, NADH dehydrogenase subunit 3; ND5, NADH dehydrogenase subunit 5; ND4, NADH dehydrogenase subunit 4; ND6, NADH dehydrogenase subunit 6; cytB, cytochrome B; ND1, NADH dehydrogenase subunit 1; 16S, large subunit ribosomal RNA; 12S, small subunit ribosomal RNA.

However, it would be premature to consider these restriction sites as species specific, because population surveys for each species have not be done to evaluate intraspecific polymorphism. One example of this problem was detected in this study, the *Bgl* II site was polymorphic between the two samples of *M. quadrifasciata*, each corresponding to different recognized subspecies (Tab. I and Fig. 1). Thus, population surveys will be necessary to evaluate the restrictions sites as potential markers for subspecies and species characterization.

The PCR-RFLP technique used here in an attempt to better localize restriction sites close to each other, also provided information about the mitochondrial gene order. By considering the gene contents of each amplified fragments (through analogy to *A. mellifera* mitochondrial genome) and the position of restriction sites on the map, the main mitochondrial genes could be positioned (Fig. 1). The same methodology was applied by Francisco et al. (2001) to determine the partial genomic map of *Plebeia* species. Comparing the genomic maps of *Plebeia*, *Melipona* and *A. mellifera* we conclude that the main mitochondrial genes present conserved position.

The gene positions added to the restriction maps permitted us to visualize that five of the seven conserved restriction sites (Fig. 1) are

located in genes that are known to be well conserved in their nucleotide sequence among animals (Simon et al., 1994): COI – three sites; cytB – one site; and 16S – one site. These five restriction sites were previously mapped at the same genomic position in *Plebeia* (Francisco et al., 2001) and *A. mellifera* (Crozier and Crozier, 1993). The two remaining conserved sites are also presented in four *Plebeia* species, except in *P. remota* (Francisco et al., 2001).

The study of Meliponini in a multidisciplinary sense is in the beginning, more data combining ecological, behavioral, and molecular characters are need to understand the biology, phylogeography, evolution and phylogeny of this important group of bees. This present paper is a contribution toward this goal. The restriction maps reported are already being used as a guide in population surveys of some species and also permitted us to infer differences in mtDNA gene order. Those inferred differences are now being investigated by sequencing and some tRNA gene transpositions have already been verified (Silvestre et al., 2002).

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Résumé – Cartes génomiques et de restriction de l'ADNmt de sept espèces de *Melipona*. La tribu des Meliponini est le groupe le plus divers des abeilles corbiculées, mais il existe peu d'études au niveau moléculaire les concernant. L'un des genres majeurs du groupe, *Melipona* Illiger, comprend environ 40 espèces. Elles se répartissent du Mexique à l'Argentine et jouent des rôles écologique et économique importants. L'analyse des polymorphismes du génome mitochondrial a été largement appliquée aux études de population ou d'évolution des abeilles et des autres organismes. Dans cette étude nous avons caractérisé l'ADNmt de sept espèces de *Melipona* : *M. bicolor*, *M. compressipes*, *M. marginata*, *M. melanoventer*, *M. quadrifasciata* (*M. q. quadrifasciata* et *M. q. anthidioides*), *M. rufiventris* et *M. subnitida.* L'ADNmt de ces espèces a été analysé avec 15 enzymes de restriction qui ont généré au total 27 sites de restriction. Le nombre total de sites de restriction a varié selon les espèces : il était le plus faible (12 sites) chez *M. rufiventris* et *M. melanoventer* et le plus élevé (19 sites) chez *M. marginata* (Tab. I). La taille entière de la molécule a été estimée à environ 18 500 paires de bases. Des cartes de restriction ont été construites pour chaque espèce (Fig. 1) et leur analyse a montré la présence de sept sites conservés au sein des espèces. On déduit de la comparaison des cartes génomiques de *Plebeia, Melipona* et *Apis mellifera* que les principaux gènes mitochondriaux présentent une position conservée.

***Melipona* / abeille sans aiguillon / ADNmt / carte de restriction / RFLP**

Zusammenfassung – Mitochondriale DNA Restriction und Genom Karten von sieben *Melipona* Arten (Apidae: Meliponini). Der Tribus Meliponini ist die unterschiedlichste Gruppe innerhalb der corbiculaten Bienen. Trotzdem gibt es von ihnen bisher kaum Untersuchungen auf molekularer Ebene. Eine der Hauptgattungen dieses Stammes, *Melipona* Illiger, besteht aus etwa 40 Arten. Ihre geographische Verbreitung reicht von Mexiko bis Argentinien und sie haben eine wichtige ökologische und ökonomische Bedeutung. Analysen der mitochondrialen Genompolymorphismen wurden allgemein für Studien zur Population und Evolution von Bienen und anderen Organismen angewendet. In dieser Untersuchung charakterisierten wir die mtDNA von 7 *Melipona* Arten: *M. bicolor*, *M. compressipes*, *M. marginata*, *M. melanoventer*, *M. quadrifasciata* (*M. q. quadrifasciata* und *M. q. anthidioides*), *M. rufiventris* und *M. subnitida*. Die mtDNA dieser Arten wurde mit 15 Restriktionsenzymen analysiert. Insgesamt wurden 27 Restriktions-Schnittstellen erzeugt. Die Gesamtzahl der Restriktions-Schnittstellen variierte zwischen den Arten, die niedrigste wurde bei *M. rufiventris* und *M. melanoventer* (12 Schnittstellen) gefunden, und die höchste bei *M. marginata* (19 Schnittstellen) (Tab. I). Die Größe des Gesamtmoleküls wurde auf annähernd 18500 bp geschätzt. Restriktionskartierungen (Abb. 1) wurden für jede Art erstellt und die Analyse dieser Kartierungen ergab 7 konservierte Regionen innerhalb der Arten. Aus einem Vergleich der Genomkarten von *Plebeia*, *Melipona* und *Apis mellifera* schließen wir, dass die hauptsächlichen mitochondrialen Gene konservierte Positionen darstellen.

***Melipona* / Stachellose Bienen / mtDNA / RFLP / Restriktionskartierung**

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