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Original article

Mitochondrial DNA characterization of five species of *Plebeia* (Apidae: Meliponini): RFLP and restriction maps

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Abstract – The present work characterized the mitochondrial DNA (mtDNA) of five species of *Plebeia* (*Plebeia droryana*, *P. emerina*, *P. remota*, *P. saiqui* and *P.* sp.) and generate a data set to be used in further populational, phylogenetic, and biogeographic studies. The mtDNA of each species was analyzed using 17 restriction enzymes and restriction maps were built. A high level of interspecific variability was found. The total size of the mtDNA was estimated to be 18500 bp. Through a combination of PCR and examination of restriction fragment length polymorphism, the locations of 14 of the main mitochondrial genes were located on restriction maps. We verified a gene order identical to *Apis mellifera*.

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

1. INTRODUCTION

The tribe Meliponini (stingless bees) exhibits a pantropical distribution and contains a great diversity of species, including key species of several Brazilian ecosystems. Some species are essential for the pollination of a great part of the Atlantic forest flora (Kerr et al., 1996). The exclusively neotropical genus *Plebeia*, morphologically one of the most primitive genera of this tribe (Michener, 1990), is considered a post-Gondwanan group that originated in the region of southeastern Brazil (Camargo and Wittmann, 1989). So far 30 species have been described across a geographic range

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from Mexico to Argentina (Michener, 2000). Given the incomplete knowledge of the taxonomy of the group, it is estimated that *Plebeia* comprises about 40 species (Moure, unpublished data). Moreover, the biology of the described species is still poorly understood. Several systematic problems are commonly described (Camargo and Pedro, 1992) and phylogenetic studies within the genus are needed.

Mitochondrial DNA (mtDNA) is one of the most widely used molecules in systematic, species characterization, population structure, and phylogenetic studies. In general, animal mtDNA is a small, circular molecule, with a high evolutionary rate and a very conserved gene order and content (Gray, 1989). One of the characteristics of mtDNA is a mix of conserved regions and others showing a high substitution rate. Analysis by restriction fragment length polymorphism (RFLP) technique allows the study of this molecule as a whole, including both conserved and variable regions.

Detailed studies of bee mtDNA are recent and primarily restricted to the genus *Apis*. *Apis mellifera* L. mtDNA is approximately 16300 base pairs (bp) long (Crozier and Crozier, 1993). Polymorphism of *Apis* mtDNA has provided markers that have been extremely important for the study of populations, hybridization, subspecies, and species (Arias et al., 1990; Sheppard et al., 1991a; Sheppard et al., 1991b; Smith et al., 1991; Garnery et al., 1992; Lobo, 1995; Arias and Sheppard, 1996; Arias et al., 1996; Sheppard et al., 1996; Meixner et al., 2000). The great volume of data generated by the analysis of this molecule in *Apis* encouraged us to apply the same techniques to related questions in other bees. As few mtDNA studies have been reported for Brazilian native bees (Costa, 1998), we report here the comparison and characterization of mtDNA from five *Plebeia* species by restriction enzyme site mapping.

2. MATERIALS AND METHODS

The *Plebeia* species studied were the following: *Plebeia droryana* (Friese), *P. emerina* (Friese), *P. remota* (Holmberg), *P. saiqui* (Friese) and *P.* sp. (known as "mirim-ponta-de-árvore"). The samples were collected from five nests (one for each species) maintained at the Laboratório de Abelhas do Departamento de Ecologia do IB-USP. All the colonies were originally from Cunha, SP, except *P. emerina*, which was from São Paulo, SP.

2.1. Restriction fragment length polymorphism (RFLP) and Southern blot

Two different procedures for total DNA extraction were applied. The TNE-protocol (Sheppard and McPheron, 1991) worked better for the species *P. remota*, *P. saiqui*, and *P.* sp. while the mini-TNE method (Arias and Sheppard, 1996) gave better results for the species *P. droryana* and *P. emerina*. All the extractions used 20 headless individuals each.

The DNA obtained by those extractions was digested overnight with the following restriction enzymes: *Bgl* II, *Eco*R I, *Hinc* II, *Hin*d III, *Pst* I, *Xba* I, *Xho* I (GIBCO BRL) and *Bam*H I, *Bcl* I, *Cfo* I, *Cla* I, *Eco*R V, *Hae* III, *Hpa* I, *Nde* I, *Pvu* II and *Sca* I (Boehringer Mannheim). Each digestion used 5 U of enzyme and 5% of the total volume of TNE extractions or 10% of the mini-TNE extractions. For double digestions, the DNA was digested with the first enzyme, precipitated, and then digested with the second enzyme.

The digests were analyzed by electrophoresis in 0.8% agarose gels, stained with ethidium bromide, observed and photographed under ultraviolet (UV) light. The DNA fragments were transferred to nylon membranes (Amersham Pharmacia) using the Southern blot technique (Sambrook et al., 1989) for further hybridization. The

probe was derived from *A. mellifera* total mtDNA, amplified through PCR (polymerase chain reaction) in 12 fragments covering the whole genome (Arias et al., 1998) and labeled with digoxigenin using the DIG DNA Labeling and Detection Kit (Boehringer Mannheim). The labeling reaction and membrane development were carried out following the manufacturer's protocol. Due to constraints of using heterologous probe, the hybridizations were conducted at 54 °C overnight.

2.2. Polymerase chain reactions (PCR)

Total DNA from single individuals was extracted using Chelex (Walsh et al., 1991), and 5 μL of the supernatant were used as template for PCR reactions. Each reaction was set up with 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) and sterile water to achieve a final volume of 50 μL. The PCR amplifications consisted of an initial denaturation of 94 °C/5 min, followed by 35 cycles of 94 °C/60 s for denaturing the DNA, 80 s at the appropriate temperature for annealing (Tab. I) and 64 °C/120 s for elongation. An additional final extension step of 64 °C for 10 min was performed. The primers were derived from *A. mellifera* (Hall and Smith, 1991; Arias et al., 1998), *Melipona bicolor* Lepeletier (present work), and other organisms (Simon et al., 1994). The PCR products were analyzed in 0.8% agarose gels, stained with ethidium bromide, visualized and photographed under UV light.

The PCR-RFLP technique (Simon et al., 1993) was also used. The PCR fragments were digested with the same restriction enzymes used for the total DNA in an attempt to find sites very close to each other and not detectable by Southern blot. The products were analyzed on 1.5% Nusieve (FMC) 3:1 agarose gels.

3. RESULTS AND DISCUSSION

The *Plebeia* species mtDNA was analyzed by RFLP using 17 restriction enzymes. Fragment sizes were calculated from Southern blot membranes (Fig. 1) and the total length of *Plebeia* mtDNA was estimated to be about 18 500 bp. This estimated size was in agreement with the reported mtDNA genomes from other animals (Brown, 1985). No length variation among the species was detected.

Four of the 17 restriction enzymes (*Cfo* I, *Hinc* II, *Hpa* I and *Pvu* II) did not cut the mtDNA of any species. Based on the five species analyzed and the 13 remaining enzymes, a total of 28 different restriction sites were generated. The enzymes *Bam*H I and *Xba* I cut the mtDNA only once and were species specific. Thirteen other restriction sites were also determined to be species specific by absence or presence. A summary of restriction site numbers generated per enzyme for each species is presented in Table II.

The total number of restriction sites per species ranged from 23 (*P. droryana*) to 16 (*P. remota*), with 11 sites conserved among the five species. Using double digestions and the Southern blot technique, we could construct initial restriction maps. However to further improve the resolution of such maps, 10 mitochondrial regions were amplified for each species (Fig. 2).

Using *A. mellifera* mtDNA sequence as a model for the position of the primers and respective amplified fragments, it was verified that approximately 13 700 bp were amplified, equivalent to about 75% of the *Plebeia* mitochondrial genome.

The PCR-RFLP fragments were initially used for the refinement of the restriction maps. As an illustrative example, the PCR product amplified by the primers AMB17 and AMB18 was approximately 1 700 bp in length. When digested with *Eco*R I, this fragment produced four fragments of 820, 450, 250, and 180 bp in length (Fig. 3)

Table I. Pairs of primers used to amplify regions of *Plebeia* mtDNA.

Pair	Name	Sequence (5' → 3')	T_a (°C)	Main genes	Al (bp)	Reference
1	mtD2	GCTAAATAAGCTAACAGGTTCAT	42	ND2, COI	2300	(Simon et al., 1994)
	mtD9	CCCGGTAAAATTAAAATATAAACTTC				(Simon et al., 1994)
2	mtD7	GGATCACCTGATATAGCATTCCC	44	COI	1600	(Simon et al., 1994)
	COI-IIR	GATCAATATCATTGATGACC				(Hall and Smith, 1991)
3	COI-IIF	TCTATACCACGACGTTATTC	44	COII	900	(Hall and Smith, 1991)
	mtD18	CCACAAATTTCTGAACATTGACCA				(Simon et al., 1994)
4	mtD19	GAAATTTGTGGAGCAAATCATAG	42	ATP8, ATP6, COIII	1700	(Simon et al., 1994)
	mtD22	TCAACAAAGTGTCAGTATCA				(Simon et al., 1994)
5	5612R	GAAATTAATATAACATGACCACC	42	COIII, ND3	1100	(present work[a])
	tPheF	GCGTAATATTGAAAATATTAATGA				(present work[a])
6	Seq18	GAACTATCAATTTGATATTG	42	ND3, ND5	2400	(present work[b])
	8467F	GGAATTTTTTTTTGAATGAA				(present work[a])
7	mtD24	GGAGCTTCAACATGAGCTTT	42	ND4, ND6	2500	(Simon et al., 1994)
	mtD28	ATTACACCTCCTAATTTATTAGGAAT				(Simon et al., 1994)
8	AMB17	TATGTACTACCATGAGGACAAATATC	42	cytB, ND1	1700	(Arias et al., 1998)
	AMB18	ATTCAGGATCGTAAAGGTCC				(Arias et al., 1998)
9	MEL 3	TAAAGTTAAAAAAGCAACTC	43	16S	800	(present work[b])
	16SF	CACCTGTTTATCAAAAACATGTCC				(Hall and Smith, 1991)
10	16SR	CGTCGATTTGAACTCAAATCATG	42	16S, 12S	1800	(Hall and Smith, 1991)
	mtD36	AAACTAGGATTAGATACCCTATTAT				(Simon et al., 1994)

[a] Derived from *Apis mellifera*; [b] derived from *Melipona bicolor*.
T_a: annealing temperature; Al: approximate length.

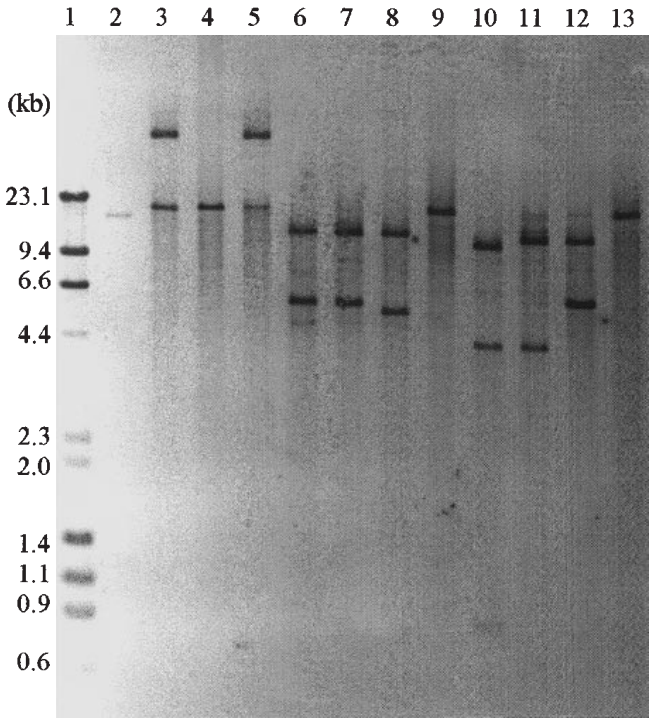

Figure 1. Membrane containing *P.* sp. mtDNA fragments (lanes 3–13). (1) Molecular weight markers λ/*Hin*d III and ϕx/*Hae* III, with lengths in kilobases. (2) *A. mellifera* mtDNA digested with *Hin*d III. (3) *Hinc* II. (4) *Bgl* II + *Xba* I. (5) *Xba* I. (6) *Xba* I + *Pst* I. (7) *Pst* I. (8) *Pst* I + *Bgl* II. (9) *Bgl* II. (10) *Bgl* II + *Eco*R I. (11) *Eco*R I. (12) *Hin*d III. (13) *Xho* I.

Table II. Number of restriction sites generated per enzyme for each species.

Enzymes	*P. droryana*	*P. emerina*	*P. remota*	*P. saiqui*	*P.* sp.
**Bam*H I (M)	1	0	0	0	0
Bcl I (B)	4	4	4	4	3
Bgl II (G)	1	2	0	2	1
Cfo I	0	0	0	0	0
Cla I (C)	2	2	2	1	3
*Eco*R I (E)	5	5	3	5	5
*Eco*R V (V)	0	0	0	1	1
Hae III (A)	1	1	1	1	1
Hinc II	0	0	0	0	0
*Hin*d III (D)	3	3	2	3	2
Hpa I	0	0	0	0	0
Nde I (N)	1	1	1	0	2
Pst I (P)	2	2	1	2	2
Pvu II	0	0	0	0	0
Sca I (S)	2	1	1	1	1
**Xba* I (X)	0	0	1	0	0
Xho I (H)	1	1	0	1	1
Total	23	22	16	21	22

* Indicates species specific enzymes. The letter in parenthesis is the abbreviation used in Figure 4.

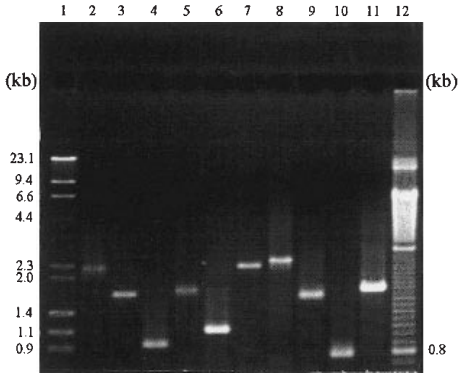

Figure 2. Agarose gel containing the 10 amplified mitochondrial fragments of *P. droryana*. (1) Molecular weight markers λ/*Hin*d III and ϕx/*Hae* III, with lengths in kilobases. (2) mtD2 + mtD9. (3) mtD7 + COI-IIR. (4) COI-IIF + mtD18. (5) mtD19 + mtD22. (6) 5612R + tPheF. (7) Seq18 + 8467F. (8) mtD24 + mtD28. (9) AMB17 + AMB18. (10) MEL3 + 16SF. (11) 16SR + mtD36. (12) 100 bp ladder with lengths in kilobases.

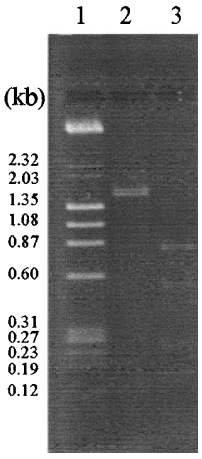

Figure 3. Analysis of the PCR fragment AMB17 + AMB18 of *P. emerina*, in a 1.5% Nusieve 3:1 agarose gel. (1) Molecular weight markers λ/*Hin*d III and ϕx/*Hae* III, with lengths in kilobases. (2) non-digested PCR fragment. (3) PCR fragment digested with *Eco*R I.

indicating the presence of three *Eco*R I sites, while the mapping by Southern blot had detected only one site.

These data also allowed us to add the relative position of the main mitochondrial genes to the restriction maps. We could infer that the *Plebeia* species here studied presented the same gene order as described in *A. mellifera*. This hypothesis was based on the simple correlation between the gene content of the amplified fragments (data from *A. mellifera* mitochondrial genome) and the mapped restriction sites (Fig. 4). Thus we could locate most of the restriction sites within genes. Ten of the 11 conserved sites mentioned above are located within the following genes: COI (three sites), cytB (one site), ND1 (three sites), 12S (two sites) and 16S (one site); one site, which corresponds to the *Hin*d III site located between the genes ND3 and ND5, could not be mapped. This region contains three tRNA genes in the honey bee and does not show any *Hin*d III site in that species, however, it presents three potential 6-base sequences that may generate *Hin*d III sites by a single transversion. From an evolutionary perspective, the 11 conserved sites must be located in mitochondrial gene regions that are under severe functional constraints, resulting in their fixation during the evolutionary history of this group of bees.

In addition to an improved understanding of restriction maps and gene structure in this group, the application of the PCR methods outlined here may be useful in the future for rapid identification of *Plebeia* species, as several sites appear to be species specific. However, more studies are needed to verify levels of intraspecific variation. Such methodology has been applied successfully to *A. mellifera* subspecies (Crozier et al., 1991; Hall and Smith, 1991; Sheppard et al., 1994).

Six of the PCR products of *Plebeia* (mtD7 + COI-IIR, COI-IIF + mtD18, mtD19 + mtD22, 5612R + tPheF, Seq18 + 8467F and AMB17 + AMB18) were slightly smaller than the corresponding products in

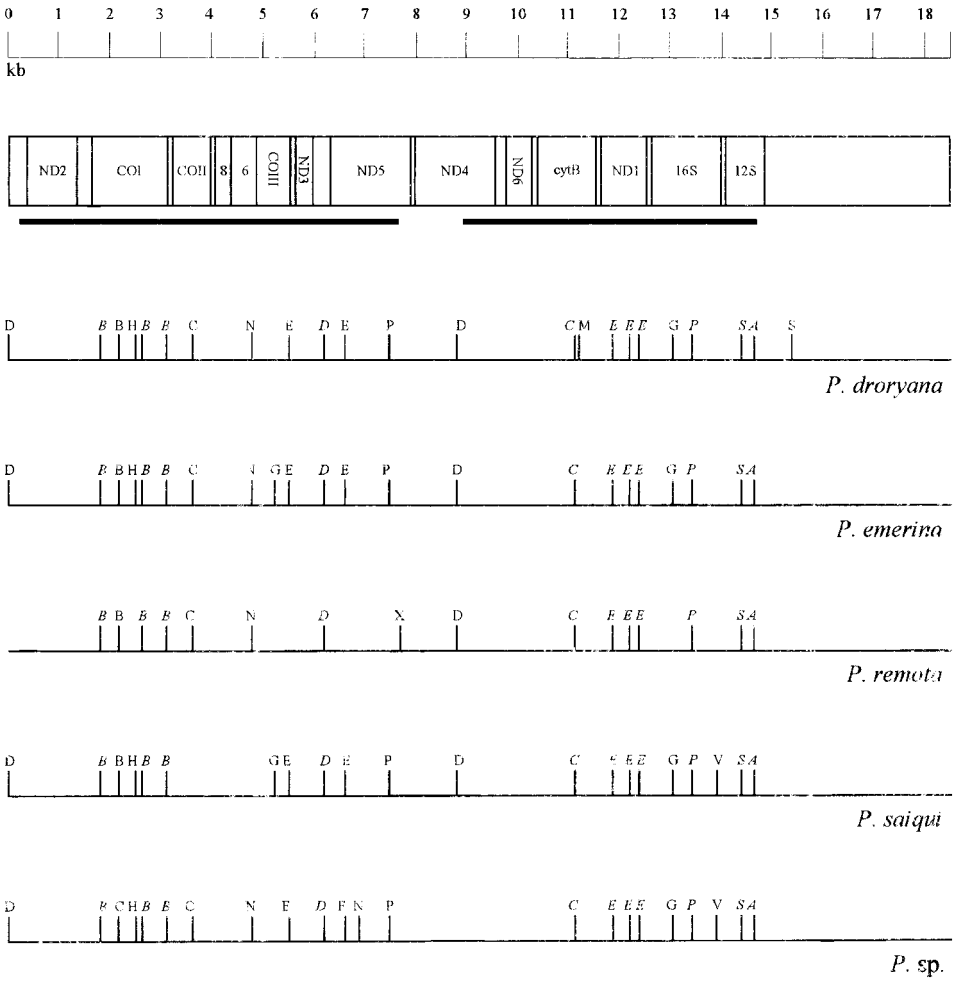

Figure 4. Linearized restriction site maps, gene positions and the PCR-amplified regions (black bars) of the 5 species of *Plebeia* mitochondrial genomes. The italicized letters refer to conserved sites. M, *Bam*H I; B, *Bcl* I; G, *Bgl* II; C, *Cla* I; E, *Eco*R I; V, *Eco*R V; A, *Hae* III; D, *Hin*d III; N, *Nde* I; P, *Pst* I; S, *Sca* I; X, *Xba* I; H, *Xho* 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.

A. mellifera, and the differences, a total of about 300 bp, are being studied. However, restriction fragments that include the A + T-rich region (hypothesis inferred from a combined data from Southern blot, PCR-RFLP gene location and comparisons to *A. mellifera* mtDNA genome) were 2500 bp longer than the same fragments in *A. mellifera*. Therefore, the total size of the *Plebeia* mitochondrial genome (18500 bp) is about 2200 bp larger than that of *A. mellifera*. Considering that mtDNA rarely

possesses repetitive DNA, transposons, introns, or pseudogenes (Gray, 1989) such difference seems to be concentrated in the non-coding A + T-rich region and also in small intergenic regions. In *Drosophila*, mtDNA length differences concentrated in the A + T-rich region are well known, ranging from 1 to 5 kb (Fauron and Wolstenholme, 1976; Fauron and Wolstenholme, 1980a; Fauron and Wolstenholme, 1980b). The *A. mellifera* A + T-rich region is about 825 bp, and, in *Plebeia* species, this region seems to be approximately 3300 bp long. This difference can explain our difficulty in amplifying this region using primers designed from other invertebrate sequences.

The present work reports the first mtDNA characterization of Meliponini and the second in the family Apidae. The basic analysis performed here will be very useful in further population and evolutionary studies of this tribe. The methodology (RFLP) appears well-suited to provide molecular markers for species identification. However, the number of species should be increased, since the genus is comprised of about 40 species. Additional data from morphology, behavior and from other molecular methods will certainly contribute to improve our understanding of the biology, biogeography, and evolution of this genus.

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Résumé – Caractérisation de l'ADNmt de cinq espèces de *Plebeia* (Apidae : Meliponini) : RFLP et cartes de restriction. Les abeilles du genre *Plebeia*, dont le centre d'origine est le sud du Brésil, jouent un rôle important en tant que pollinisateurs de la flore indigène. Comme pour la plupart des abeilles sans aiguillon, de nombreux aspects de la biologie et de l'évolution des espèces de *Plebeia* n'ont pas été étudiés. Cette étude vise à un début de caractérisation de l'ADNmt de cinq espèces du genre *Plebeia* (*P. droryana, P. emerina, P. remota, P. saiqui* et *P*. sp.) et à produire un ensemble de données qui pourra être utilisé dans d'autres études de population, de phylogénétique et de biogéographie.
L'ADNmt de chaque espèce a été extrait et analysé par digestion simple et double à l'aide de 17 enzymes de restriction. On a trouvé une variabilité interspécifique élevée. Au total 28 sites de restriction ont été cartographiés, dont 11 sont présents chez toutes les espèces. Le nombre de sites varie de 23 chez *P. droryana* à 16 chez *P. remota* (Tab. II). On estime la taille totale de l'ADNmt à environ 18500 bp. Des cartes de restriction ont été construites pour chaque espèce. À l'aide de la technique PCR-RFLP (Fig. 3), plusieurs sites de restriction ont été situés sur des gènes spécifiques. Ainsi, la localisation de 14 des principaux gènes mitochondriaux a été ajoutée aux cartes de restriction (Fig. 4). L'ordre génomique de ces gènes est identique à celui de l'abeille domestique, *Apis mellifera*.

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

Zusammenfassung – Charakterisierung der Mitochondrien DNA von 5 *Plebeia* Arten (Apidae, Meliponini): RFLP und Kartierung der Restriktionsstellen. Bienen der Gattung *Plebeia*, deren evolutionärer Ursprung vor allem in Südbrasilien liegt, spielen eine wichtige Rolle bei der Bestäubung der einheimischen Flora. Wie bei den

meisten stachellosen Bienen sind unzählige Aspekte der Biologie und der Evolution bei den *Plebeia* Arten nicht untersucht. Diese Untersuchung soll der Beginn für eine Charakterisierung der mt-DNA der 5 Arten *Plebeia droryana*, *P. emerina*, *P. remota*, *P. saiqui* und *P.* sp. sein und eine Datenbasis erstellen, die als Grundlage für weitere Untersuchungen über Populationen, Phylogenie und Biogeografie dient. Die mt-DNA wurde extrahiert und durch einfache oder doppelte Verdauung durch 17 Restriktionsenzyme analysiert. Die zwischenartliche Variabilität war hoch. Insgesamt wurden 28 Restriktionsstellen kartiert, von denen 11 bei allen Arten vorkamen. Die Zahl der Restriktionsstellen variierte von 23 bei *P. droryana* bis 16 bei *P. remota* (Tab. II). Die Gesamtlänge der mt-DNA wurde auf 18 500 Basenpaare geschätzt. Für jede Art wurde ein Restriktionsmuster erstellt. Durch die PCR-RFLP Technik (Abb. 3) wurden mehrere Restriktionsstellen spezifischen Genen zugeordnet. Auf diese Weise konnten 14 der bedeutenden mitochondrialen Gene in das kartierte Restriktionsmuster eingefügt werden. Die Anordnung dieser Genen ist identisch mit der von *Apis mellifera.*

***Plebeia* / Stachellose Bienen / mt-DNA / RFLP / Restriktionsmuster**

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