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Original article

Intraspecific variation in the stingless bee *Melipona beecheii* assessed with PCR-RFLP of the ITS1 ribosomal DNA*

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Abstract – In previous works, significant variation in morphometric and molecular characteristics was detected among populations of *M. beecheii*. Here RFLP tests of the internal transcribed spacer 1 of the ribosomal gene were performed to confirm those results and to evaluate the intraspecific variability within the species. The complete ITS1 region and the flanking regions showed length variation (1720 to 1670) and also three different restriction patterns that allowed differentiation of three groups of colonies with different geographic distribution. Mexican colonies from Yucatán, Campeche and Chiapas, together with one colony from northern Guatemala formed one group, a second was composed of colonies from southern Guatemala, El Salvador and Costa Rica and a third one corresponded to one colony from San Marcos (Guatemala but close to the Mexican border). Such test could be used to characterize locally adapted ecotypes subject to conservation efforts.

stingless bees / *Melipona beecheii* / ITS1 region / RFLP / genetic variability

1. INTRODUCTION

The stingless bee *Melipona beecheii* (Bennett, 1831) is native of the tropical region of the American continent and has a natural distribution ranging from Mexico to Costa Rica (Ayala, 1999). In Mexico, the breeding and management of this stingless bee (known in the Mayan language as "xunan kab" or "colel kab") has been practiced in the Yucatán Peninsula since the time of the Maya civilization (Weaver and Weaver, 1981; González-Acereto and De Araujo, 2005; González-Acereto, 2008). This bee builds its nests in the trunks of living trees and is exploited for honey production. In addition, the cultivation of stingless bees represents an advantage for many species of native and endemic flora that are mainly pollinated by native bees (Heard, 1999). At present, *M. beecheii* populations are under threat due to the intense degradation of the Yucatecan forest (González-Acereto, 1999; Quezada-Euán et al., 2001; Villanueva et al., 2005).

In spite of their ecological and cultural importance, there are few molecular studies on the genus *Melipona*. Recently, Fernandes-Salomão et al. (2005) have fully characterized the first internal transcribed spacer (ITS1) of the ribosomal gene (rDNA) in three *Melipona*

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Table I. Sampling localities and RFLP-ITS1 pattern distribution of the specimens of *M. beecheii* analyzed (N = number of colonies analysed).

Country	State	Locality	N	ITS1-A	ITS1-B	ITS1-C
México	Yucatán	Mérida	42	42		
	Chiapas	Chiapas	4	4		
	Campeche	Tankunché	14	14		
Guatemala	Petén	Santa Elena	2	2		
	Retalhuleu	Salamá	1		1	
	Alta Verapaz	Caquiqual	2		2	
		Carchá	1		1	
	San Marcos	Pajapita	1			1
	Chiquimula	El Jocotal	1		1	
		Esquipulas	1		1	
	Santa Rosa	Pueblo Nuevo Viñas	1		1	
El Salvador	Chalatenango	San Ignacio	31		31	
Costa Rica	Heredia	Heredia	1		1	
	Guanacaste	Juntas de Abangares	2		2	
		Cañas	1		1	
	Alajuela	Sabana Larga	1		1	
		Barrio Jesús	1		1	
		Atenas	1		1	
	Puntarenas	Miramar	2		2	
Total				62	47	1

species distributed in Brazil. Due to the long size of this region (around 1400 bp), a shorter fragment (400–500 bp) was PCR-amplified in eight *Melipona* species and its phylogenetic utility was confirmed. The usefulness of the ITS1 region has been also determined for intraspecific studies in the Brazilian species *M. subnitida* (Cruz et al., 2006). In that work, partial 3' sequences of the ITS1 region (654 bp) of samples from 13 locations of the Northeast of Brazil were sequenced. A marked variation was observed in the specimens analyzed, related to the ancient origin of this species and the low rates of gene flow among the populations. Despite these conclusions, no correlation was observed between the ITS1 divergence and the geographical distance of the sampled localities, suggesting that the *M. subnitida* specimens may correspond to isolated populations.

In the present work, intraspecific sequence variation in the ITS1 region was assessed through a RFLP (restriction fragment length polymorphism) approach in *M. beecheii* specimens sampled from different localities in Mexico, El Salvador, Guatemala and Costa Rica. The information depicted from the molecular analysis could be relevant to characterize the different populations and to establish appropriate protection and conservation strategies as some of them could be considered as locally adapted ecotypes.

2. MATERIALS AND METHODS

2.1. Sample collection

Samples of worker *M. beecheii* were collected in Mexico (Yucatán Peninsula, Campeche and Chiapas) and in Guatemala, El Salvador and Costa Rica. The collection sites and number of sampled colonies are listed in Table I. Each sample consisted on 10–20 adult worker bees collected from the inner nest of each colony. All samples were preserved in absolute ethanol at –20 °C until used for the molecular analyses.

2.2. DNA extraction and PCR amplification

Three legs were dissected from one bee per colony and air dried to eliminate the ethanol. Total

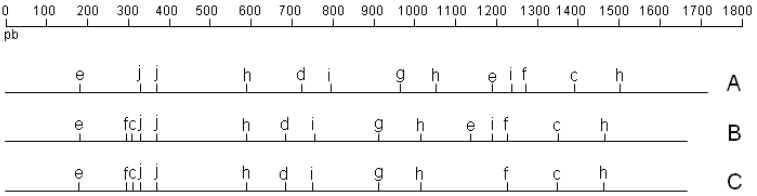

Figure 1. Restriction site maps of the three patterns (ITS1-A, ITS1-B and ITS1-C) found in the ITS1 region of *M. beecheii*. The restriction enzymes have been named with lower case letters (c = *Esp*3I, d = *Fok*I, e = *Hph*I, f = *Nru*I, g = *Nci*I, h = *Tas*I, i = *Tsp*45I and j = *Sac*II).

genomic DNA was extracted using the DNeasy® tissue kit (QIAGEN) following manufacturer instructions. The total dilution volume was 100 µL. Four µL of DNA template were taken for the PCR amplification.

Primers used for the amplification of the complete ITS1 region were cas18sf1 and cas5p8sB1d (Ji et al., 2003). The amplification reactions were performed in 25 µL volume with PureTaq™ Ready-To-Go™ PCR beads (GE Healthcare) in a PTC-200 Thermal Cycler (Biorad). PCR conditions involved an initial denaturation step at 96 °C for 5 min followed by 34 cycles of 96 °C for 45 s, 60 °C for 1 min, 72 °C for 1 min. The last cycle was followed by a final extension of 72 °C for 10 min. The amplified fragments were analyzed by electrophoresis in 1.5% agarose gels stained with ethidium bromide.

2.3. RFLP analyses

Amplified ITS1 region of one worker bee of each sampled colony was digested with the following restriction enzymes: *Eco*RI, *Eco*RV, *Esp*3I, *Fok*I, *Hph*I, *Nru*I, *Nci*I, *Tas*I, *Tsp*45I and *Sac*II. The reactions were conducted at 37 °C, except for the enzyme *Tas*I (65 °C), in a PTC-200 Thermal Cycler (Biorad) for 16 hours. The digested products were electrophoresed in 2% agarose gels, stained with ethidium bromide and documented under UV light. Restriction maps were inferred from the RFLP patterns.

3. RESULTS

The total size of the PCR amplified region (including the partial 18.5S and 5.8S and the complete ITS1) in the *M. beecheii* specimens varied from ca. 1720 bp observed in those colonies located in Mexico and northern Guatemala to ca. 1670 in those from southern Guatemala, El Salvador and Costa Rica.

Two of the restriction enzymes used in the RFLP assays did not cut the ITS1 region of any of the *M. beecheii* specimens (*Eco*RI and *Eco*RV). The other eight enzymes presented different number of restriction sites ranging from three (enzyme *Tas*I) to one (enzymes *Fok*I and *Nci*I) (Fig. 1).

With the enzymes *Nru*I and *Esp*3I two groups of colonies could be recognized, one formed by the colonies from Mexico and northern Guatemala and a second one by the colonies from southern Guatemala, El Salvador and Costa Rica. With the enzymes *Hph*I and *Tspe*45I the colony from San Marcos located in Guatemala, but close to the Mexican border, could de differentiated from the other two groups that were almost indistinguishable despite a slight size difference in one of the bands due to the difference in the size of the amplified region (Fig. 2). The other enzymes did not show significant differences in the restriction patterns among the *M. beecheii* colonies of different origins. Therefore three restriction patterns were obtained that showed different geographical distribution: ITS1-A was observed in colonies from Mexico and northern Guatemala, ITS1-B ranged from southern Guatemala, El Salvador to Costa Rica and ITS1-C was restricted to the colonies from San Marcos in Guatemala but close to the Mexican border (Fig. 3).

4. DISCUSSION

The size of the PCR amplified region obtained in the specimens of *M. beecheii* varied

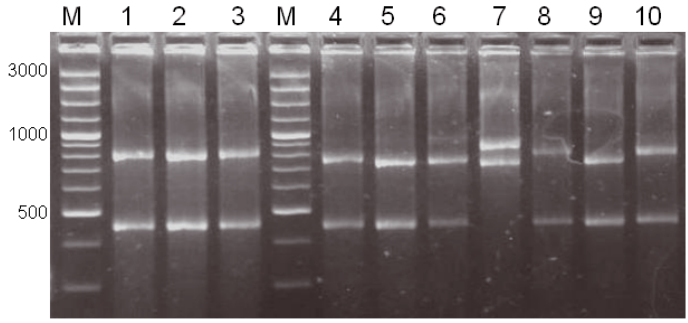

Figure 2. RFLP patterns obtained with the enzyme *Tsp*45I. The pattern ITS1-A was observed in the specimens of *M. beecheii* from México (Yucatán, Campeche and Chiapas positions 1, 2 and 3 respectively) and also in northern Guatemala (Petén, positions 8 and 10), the pattern ITS1-B has been observed in specimens from El Salvador (4) and southern Guatemala (Alta Verapaz, Chiquimula and Retalhuleu in positions 5, 6 and 9 respectively), the pattern ITS1-C (position 7) has been only detected in San Marcos (Guatemala). M is the GeneRuler 100bp DNA ladder plus (Fermentas).

Figure 3. Geographical distribution of the RFLP patterns ITS1-A, ITS1-B and ITS1-C found in *M. beecheii*.

from ca. 1720 to ca. 1670 so, excluding the flanking conserved regions of the 18S and 5.8S genes, the total size of the complete ITS1 region was about 1520 bp in the Mexican and northern Guatemala specimens, 1469 in those from southern Guatemala, El Salvador and Costa Rica and 1457 in the specimens from San Marcos (Guatemala). The size is within the range observed in other species of the genus *Melipona*. Fernandes-Salomão et al. (2005) reported a size of 1391 bp in *M. quadrifasciata*, 1387 bp in *M. mandacaia* and 1417 bp in *M. scutellaris*. Another Brazilian stingless bee, the endemic *M. subnitida*, showed intraspecific variation ranging from 1445 to 1514 bp including the flanking conserved regions (Cruz et al., 2006). Within the Hymenoptera, larger sizes as those

reported here have been also found in the egg parasitoids of the genus *Trichogramma* (1300–1350 bp, Sappal et al., 1995). On the other hand within the family Apidae an extreme variation in the size of the ITS1 region has been observed as in the species *Anthophora abrupta* the estimated size was 83 bp (Sheppard and McPheron, 1991), in the *Apis mellifera* subspecies this region is 132 bp long (De la Rúa et al., 2007) and in *Bombus lapidarius* is also small (289 pb, Ji et al., 2003). A similar situation of extreme length and length variation has been detected in the ITS1 region of ladybird beetles of the family Coccinellidae (von der Schulenburg et al., 2001); in this case the size of the ITS1 region ranged in length from 791 to 2572 bp. This difference was attributed to the presence of repetitive elements, as has also been reported for the ITS1 region of *Melipona* species (Fernandes-Salomão et al., 2005). These repetitive elements usually appeared at the middle of the sequence and are free of functional constraints.

In this work *M. beecheii* was characterized by three restriction patterns (ITS1-A, ITS1-B and ITS1-C) congruent with the geographic distribution of the samples. Those colonies located in the Mexican states (Yucatán, Campeche and Chiapas) and at the northern side of Guatemala (Petén) were characterized by a longer length and the RFLP pattern ITS1-A, whereas those colonies from the southern part of Guatemala to Costa Rica showed smaller length and the RFLP pattern ITS1-B. The colony from San Marcos (Guatemala but close to the Mexican border) showed an intermediate length and a third RFLP pattern named ITS1-C. These results fully agree with the variation previously reported in the ITS2 region of the same samples (De la Rúa et al., 2007) and also with that observed in other samples from México (Yucatán) and Costa Rica that were analysed with other molecular markers (mitochondrial cox1 gene and microsatellites) and morphometric characters (Quezada-Euán et al., 2007). Carrillo et al. (2001) found differences between populations of *M. beecheii* from Chiapas and Yucatán using the face coloration of the clypeus and malar area, but such differentiation was not seen with the molecular marker described in the present study.

Given the length and the complex sequence of the ITS1 region, RFLP analyses have proven to be a fast and inexpensive approach to study variability of the genus *Melipona* at both inter and intraspecific levels. Fernandes-Salomão et al. (2002) used four restriction enzymes and eleven *Melipona* species collected in Brazil in an attempt to clarify the phylogenetic relationships within the group. Their results were comparable to those obtained with RFLP of the entire mitochondrial molecule and the cox1-cox2 genes, and also electrophoretic profiles and karyotype analysis. Our data support the usefulness of the ITS1-RFLP approach at the intraspecific level.

The extensive distribution of *M. beecheii* encompasses diverse environmental conditions and associated habitat types. Therefore, it is possible that many locally adapted ecotypes have arisen (Morrone, 2006). Our results give additional evidence to further support genetic differences between geographically isolated populations of *M. beecheii* (Camargo et al., 1988; Quezada-Euán et al., 2007). The extent of the morphological differences of *M. beecheii* from Belize, the Yucatán Peninsula and the Greater Antilles (Cuba) may justify their placement in a separate subspecies, *M. b. fulvipes*, different from its sister species, *M. b. beecheii* present elsewhere in Central America (Camargo et al., 1988). The distribution of ecotypes in our work seems in agreement with the former hypothesis but the analyses of more populations are needed in order to confirm these differences. It is also interesting that in spite that *M. beecheii* was the only widely used species for stingless beekeeping across Mesoamerica (Quezada-Euán et al., 2001, González-Acereto, 2008), molecular differences are still evident between geographic regions which suggest that little movement of colonies was conducted between localities by the Mayan. Alternatively, the present distribution of ecotypes may reflect a human movement of colonies only between certain regions (Chiapas and Yucatán but not into or from Central America). Stingless bees show reduced vagility, thus, they may have had difficulties in naturally dispersing further

away from the localities where they were established by the Mayan. Geographic barriers such as the mountain chain of the Sierra Madre del Sur may have stopped further contact between populations north and south of this barrier.

The genus *Melipona* probably dispersed across a proto-Antillean archipelago between South America and Mexico before the Panama bridge arouse in the Miocene (Camargo et al., 1988). In Central America, vicariance has been proposed to explain the disjunctive distribution of sister *Melipona* species. Similar events may also explain the separation of *M. beecheii* in morphologically and genetically different ecotypes.

In summary, the ITS1 molecular data revealed sufficient genetic variation within *M. beecheii* to associated polymorphisms with geographic origin. Furthermore, the ITS1 information obtained by PCR-RFLP analyses was concordant with the population differentiation estimated using morphological and molecular (mitochondrial and microsatellite) criteria. The inferences in this study are based on sequence data from a singular region in the rDNA array and may be distorted by unknown selective constraints. To produce a more representative analysis of the utility of this marker for the phylogeny and molecular characterization of these endangered bees, it will be necessary to analyse additional *Melipona* species and other mitochondrial and/or nuclear genes together with morphological comparisons.

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Variation intraspécifique chez l'abeille sans aiguillon *Melipona beecheii* déterminée par PCR-RFLP de la région ITS-1 de l'ADN ribosomal.

abeille sans aiguillon / Apidae / Meliponini / variabilité génétique / génétique population

Zusammenfassung – Bestimmung der intraspezifischen genetischen Variabilität bei der Stachellosen Biene *Melipona beecheii* mittels PCR-RFLP der ITS1 ribosomalen DNA. Molekulare Analysen an *Melipona beecheii*, einer in den amerikanischen Tropen einheimischen Stachellosen Biene, ermöglichten die Charakterisierung der Populationen über das gesamte Verbreitungsgebiet von Mexiko bis Costa Rica (Tab. I). Als molekularen Marker wählten wir die interne transkribierte Region 1 eines ribosomalen Gens. Dieser Marker wurde bereits zur populationsgenetischen Charakterisierung von drei brasilianischen *Melipona*-Arten eingesetzt (Fernandes-Salomão et al., 2005) und erwies sich auch als nützlich in intraspezifischen Untersuchungen der brasilianischen Art *M. subnitida* (Cruz et al., 2006). Die ITS1-Region der von uns untersuchten Art erwies sich als besonders komplex und lang (1670-1720 Basenpaare), so dass einfache Analysen zu Restriktionsfragment-Längenpolymorphismen (RFLP) möglich waren. Von den zehn verwendeten Restriktionsenzymen erwiesen sich vier als besonders geeignet und erlaubten die Trennung von drei RFLP-Mustern (Abb. 1 und 2). Die geographische Verteilung der Kolonien, von denen die Proben stammten, stimmte mit der Verteilung der unterschiedlichen RFLP-Muster überein und bestätigte damit die Unterschiedlichkeit von drei Populationen (Abb. 3). Diese können deshalb als an jeweils lokale Umweltbedingungen angepasste Ökotypen aufgefasst werden. Die Kolonien aus Mexiko (Yucatán, Campeche und Chiapas) bildeten zusammen mit den Kolonien des nördlichen Guatemala eine gemeinsame Gruppe, eine zweite umfasste Völker aus dem Süden Guatemalas, aus El Salvador und Costa Rica, und eine dritte Gruppe bildeten Völker aus San Marcos (eine nahe der mexikanischen Grenze gelegenen Region Guatemalas). Da wilde *Melipona*-Populationen von einer Habitatfragmentierung der Wälder stark betroffen sein können, bildet die genetische Information aus den RFLP-Analysen der ITS1 Region der *M. beecheii* Völker eine Grundlage für die Etablierung von Managementstrategien und Schutzmassnahmen für die Erhaltung dieser Art.

Stachellose Biene / *Melipona beecheii* / ITS1 Region / RFLP / Genetische Variabilität

REFERENCES

Ayala R. (1999) Revisión de las abejas sin aguijón de México (Hymenoptera: Apidae: Meliponini), Folia Entomol. Mex. 106, 1–123.

Bennett E.T. (1831) Some accounts of the habits of a mexican bee, in: Beechey F.W. (Ed.), Narrative of a voyage to the Pacific and Bering's Strait, to co-operate with the Polar Expeditions, Vol. 2, London, UK, pp. 357–365.

Camargo JMF, Moure J.S, Roubik D.W. (1988) *Melipona yucatanica* New Species (Himenoptera: Apidae: Meliponinae): stingless bee dispersal across the Caribbean Arc and Post-Eucene Vicariance, Pan-Pacific Entomol. 64, 147–157.

Carrillo A., Quezada-Euán J.J.G., Moo-Valle H. (2001) Estudio preliminar sobre la variabilidad morfológica de *Melipona beecheii* (Apidae: Meliponini) en su rango de distribución de México, América Central y el Caribe, in: Quezada-Euán J.J.G., May-Itzá W. de J., Moo-Valle H., Chab-Medina J.C. (Eds.), II Seminario Mexicano sobre abejas sin aguijón, Mérida Yucatán, México, pp. 73–78.

Cruz D.O., Jorge D.M.M., Pereira J.O.P., Torres D.C., Soares A.E.A., Freitas B.M., Grangeiro T.B. (2006) 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, Apidologie 37, 376–386.

De la Rúa P., Fuchs S., Serrano J. (2007) A scientific note on the ITS-1 region of *Apis mellifera* subspecies, Apidologie 38, 378–379.

De la Rúa P., May-Itzá W. de J., Serrano J., Quezada-Euán J.J.G. (2007) Sequence and RFLP analysis of the ITS2 ribosomal DNA in two Neotropical social bees, *Melipona beecheii* and *Melipona yucatanica* (Apidae: Meliponini), Insectes Soc. 54, 418–423.

Fernandes-Salomão T.M., Muro-Abad J.I., Campos L.A.O., Araújo E.F. (2002) Mitochondrial and nuclear DNA characterization in *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.

González-Acereto J.A. (1999) La meliponicultura yucateca en crisis: una actividad indígena a punto de desaparecer, 1er Seminario Nacional sobre Abejas sin Aguijón, Boca del Río Ver, México, pp. 9–12.

González-Acereto J.A. (2008) Cría y manejo de abejas nativas sin aguijón en México. Impresiones Planeta, 177 p.

González-Acereto J.A., De Araujo F.Ch. (2005) Manual de Meliponicultura Mexicana, Universidad Autónoma de Yucatán, Facultad de Medicina Veterinaria y Zootecnia, Fundación Produce Guerrero A.C. Imp Gramma, 46 p.

Heard T. (1999) The role of stingless bees in crop pollination, Annu. Rev. Entomol. 44, 183–206.

Ji Y., Zhang D., He L. (2003) Evolutionary conservation and versatility of a new set of primers for amplifying the ribosomal internal transcribed spacer regions in insects and other invertebrates, Mol. Ecol. Notes 3, 581–585.

Morrone J.J. (2006) Biogeographic areas and transition zones of Latin America and the Caribbean Islands based on panbiogeographic and cladistic analyses of the entomofauna, Annu. Rev. Entomol. 51, 467–494.

Quezada-Euán J.J.G., May-Itzá W. de J., González-Acereto J.A. (2001) Meliponiculture in Mexico: Problems and perspectives for development, Bee World 82, 160–167.

Quezada-Euán J.J.G., Paxton R.J., Palmer K.A., May-Itzá W. de J., Tay W.T., Oldroyd B.P. (2007) Morphological and molecular characters reveal differentiation in a Neotropical social bee, *Melipona beecheii* (Apidae: Meliponini), Apidologie 38, 247–258.

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.

Sheppard W.S., McPheron B.A. (1991) Ribosomal DNA diversity in Apidae, in: Smith D.R. (Ed.), Diversity in the Genus *Apis*, Westview Press, Boulder, Colorado pp. 89–102.

Villanueva G.R., Roubik D.W., Collí-Ucán W. (2005) Extinction of *Melipona beecheii* and traditional beekeeping in the Yucatán peninsula, Bee World 86, 35–41.

Von der Schulenburg J.H.G., Hancock J.M., Pagnamenta A., Sloggett J.J., Majerus M.E.N., Hurst 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.

Weaver N., Weaver E.C. (1981) Beekeeping with the stingless bee *Melipona beecheii* by the Yucatecan Maya, Bee World 62, 7–19.