



## RESEARCH ARTICLE - BEES

### Utility of the ITS1 Region for Phylogenetic Analysis in Stingless Bees: a Case Study of the Endangered *Melipona yucatanica* Camargo, Moure and Roubik (Apidae: Meliponini)

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#### Abstract

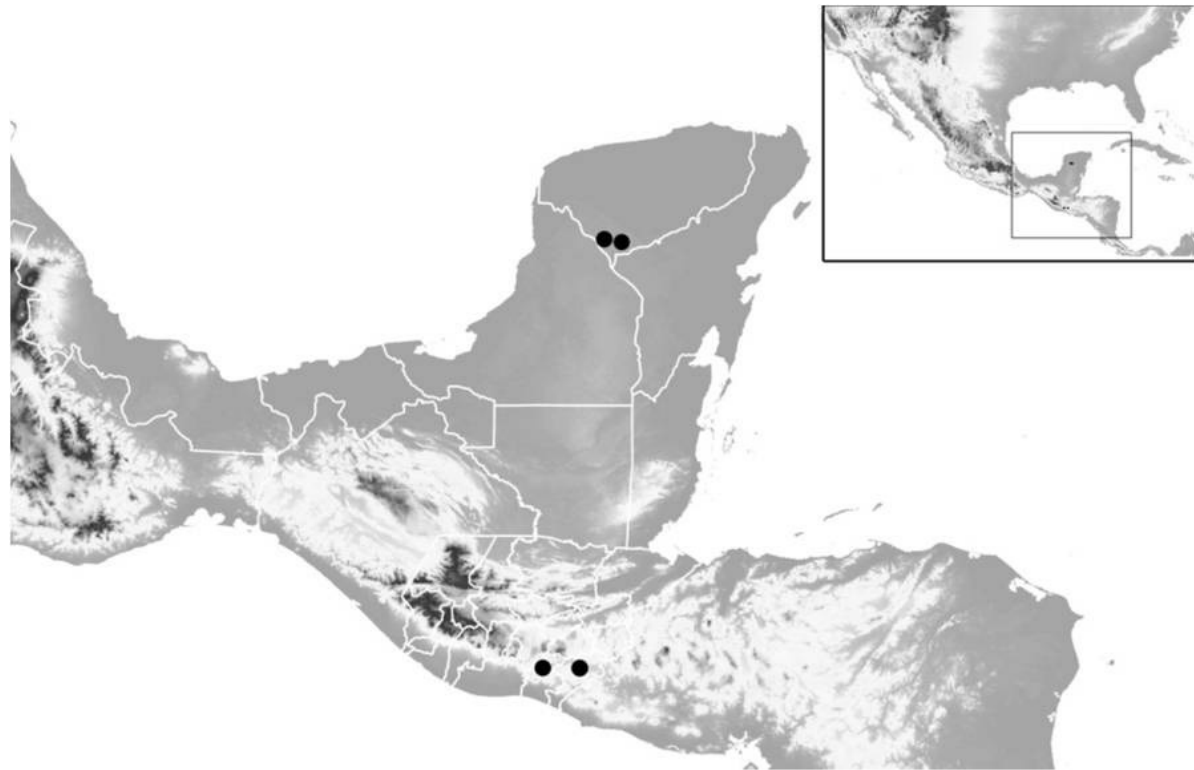
The internal transcribed spacers of the ribosomal RNA gene have been recently proposed as an appropriate marker for genetic analysis of the molecular variation of stingless bees. Herein we report the characterization of the complete ITS1 region in two populations (from Mexico and Guatemala) of the endangered *Melipona yucatanica* Camargo, Moure and Roubik. Phylogenetic analyses showed low genetic variation between populations but defined a geographic structure with Mexican and Guatemalan specimens forming two well supported clades. Low ITS1 genetic variation found between populations contrasts with high genetic variation found in other markers. Phylogenetic analysis corroborates the inclusion of *M. yucatanica* within the subgenus *Melipona sensu stricto* based on previous morphological studies. The results highlight the utility of the ITS1 for the characterization of stingless bee populations.

#### Introduction

Molecular studies addressing the stingless bees are needed not only to support preliminary morphology-based hypotheses about phylogeny, population dynamics, species delimitation and evolution, but also to establish the basis of conservation programs for this group of bees (Arias et al., 2006). Towards these ends, a phylogeny of the stingless bee genus *Melipona* has been published (Ramírez et al., 2010) based on a multigene data set (nuclear: EF1- $\alpha$ , ArgK and RNA Pol-II genes; mitochondrial: cox1 and rRNA genes: *16S*) approach. In that study 35 out of the 50 *Melipona* species known-to-date were grouped in four subgenera previously described with morphological and ecological characters (Camargo & Pedro, 2007), but still some *Melipona* species remain unassigned to a specific subgenus.

Other suitable markers for phylogeny and population genetic analyses are the internal transcribed spacers (ITS1 and ITS2) of the ribosomal genes (rRNA). Concerted evolution may have led to the homogeneity of repeat motifs within the ITS regions (Hillis & Dixon, 1991). Therefore, they have been successfully applied for population and phylogenetic studies of some stingless bee species. In particular, in the tribe Meliponini, Fernandes-Salomão et al. (2005) determined the complete ITS1 sequence from three *Melipona* species and used partial sequences of eight species to infer its phylogenetic relationships. At the intraspecific level, the ITS1 region has demonstrated its usefulness for population analysis in the Mexican species *Melipona beecheii* Bennett (May-Itzá et al., 2009; 2012) and also in two Brazilian species: *Melipona subnitida* Ducke (Cruz et al., 2006) and *Melipona quinquefasciata* Lepeletier (Pereira et al., 2009).





**Fig 1.** Study area and sampling localities of *Melipona yucatanica* in Mesoamerican region.

The Mexican stingless bee *Melipona yucatanica* Camargo, Moure and Roubik is a small bee (8 mm in average) with less than 200 worker bees per colony (Camargo et al., 1988). This species is associated with primary forest, and based on its patched distribution, various authors have suggested a recent fragmentation of *M. yucatanica* populations due to massive deforestation (Camargo et al. 1988; Ayala, 1999). Preliminary molecular studies in Mexican and Guatemalan populations of *M. yucatanica* yielded different RFLP patterns in the ITS2 region (De la Rúa et al., 2007), suggesting allopatric speciation in populations geographically separated. Furthermore, morphometric and Bayesian analyses of the mitochondrial *cox1* region and microsatellite loci revealed geographic differences between Guatemalan and Mexican populations, suggesting that *M. yucatanica* from México and Guatemala could represent two distinct species (May-Itzá et al., 2010).

Because of the rapid disappearance of its habitat and the difficulty to multiply colonies domestically, conservation measures to preserve *M. yucatanica* are urgently needed (May-Itzá et al., 2010). The taxonomic position of this species based on morphological comparative studies have placed this species within the subgenus *Melipona s. str.* (Camargo & Pedro, 2007), in relation to other South-American species (Moure et al., 2007). However its taxonomic position has not been assessed on molecular grounds yet.

Herein, the entire ITS1 region of *M. yucatanica* has been sequenced and compared with available ITS1 (complete or 3' end) from other *Melipona* species as well as with previous results on *M. yucatanica* based on morphometry and molecular

markers (De la Rúa et al., 2007; May-Itzá et al., 2010). We aimed to i) analyze the molecular diversity underlying this marker, and ii) test its phylogenetic signal to verify whether this species is included within the morphologically described subgenus *Melipona s. str.* as suggested by Camargo and Pedro (2007).

## Material and Methods

### Sampling

Feral colonies of *M. yucatanica* are difficult to find since they are restricted to preserved rain-forest, so we were only able to sample worker bees (10-20 worker bees per colony) from managed colonies located in two geographically distant Mesoamerican regions where they naturally occur: seven in México and four in Guatemala (Table 1, Fig 1). These locations showed notable climatic and geological differences: the Pacific region of Guatemala is a mountainous region, whereas the Atlantic-Caribbean region of México is a flat limestone area without mountainous zones. One specimen of each colony has been deposited in the Insect Collection of the Department of Zoology and Physical Anthropology (University of Murcia, Spain).

### DNA extraction, amplification and sequencing

DNA was extracted from two worker bees per colony using the DNeasy tissue kit (QIAGEN). The ITS1 region was amplified using the primers *cas18sf1* and *cas5p8sB1d*

**Table 1.** Sampling localities of *Melipona yucatanica* specimens included in the study, geographical information and accession numbers of sequences in GenBank.

Taxon ID	Accession number	Country	State	Locality	Geographical coordinates	Altitude
MyucMer1	HQ651727	México	Yucatán	López Portillo	19.9351278 N -89.1879278 W	142 m
MyucMer2	KF564809					
MyucMer3	KF564810					
MyucMer4	KF564811					
MyucMer5	KF564812	México	Yucatán	Huntochac	19.8061861 N -89.5007389 W	80 m
MyucMer6	KF564813					
MyucMer7	KF564814					
MyucGuat1	HQ651726	Guatemala	Jutiapa	Jutiapa	14.2980778 N -89.8970972 W	898 m
MyucGuat2	KF564806					
MyucGuat4	KF564807	Guatemala	Santa Rosa	Barberena	14.3072639 N -90.3564139 W	1220 m
MyucGuat5	KF564808					

based on the conserved sequences of the 18S and 5.8S rDNA respectively (Ji et al., 2003). The amplification program consisted of 5 min. at 96 °C, 34 cycles of 45 sec. at 96 °C, 1 min. at 60 °C and 1 min. at 72 °C, and a final extension of 10 min. at 72 °C. PCR reactions were performed with PCR beads PureTaq™ Ready-To-Go™ (GE Healthcare) in a thermocycler PTC-200 (Biorad). PCR products were purified with QIAquick PCR purification kit (QIAGEN) before directly sequencing. Sequencing was performed in both directions with the same PCR primers in an ABI 3730 DNA analyzer (Applied Biosystems) at the sequencing company Secugen S. L. (Madrid, Spain). ITS1 sequences edited with the program MEGA 5 (Tamura et al., 2011) were deposited in Genbank with the accession numbers HQ651726 (*M. yucatanica* from Guatemala) and HQ651727 (*M. yucatanica* from México).

#### Data analyses

The ITS1 datasets used included the complete ITS1 gene for *M. yucatanica*, *M. beecheii* and *Melipona quadrifasciata* Lepeletier and partial sequences of ITS1 3' end for other nine *Melipona* species obtained from Genbank (Fernandes-Salomão et al., 2005; Cruz et al., 2006; Pereira et al., 2009). In order to compare ITS1 results with other markers, a multiple-gene approach was carried out. Sequence data of mitochondrial *cox1* were obtained from Genbank for *M. yucatanica*, and for the species with available ITS1 sequences (namely, *M. quinquefasciata*, *M. beechei*, *Melipona compressipes* Fabricius, *M. quadrifasciata*, *Melipona mandacaia* Smith, *Melipona bicolor* Lepeletier, *Melipona marginata* Lepeletier, *Melipona rufiventris* Lepeletier and *Melipona scutellaris* Latreille). Separated and combined analyses were carried out.

ITS1 sequences were aligned with MAFFT software (<http://mafft.cbrc.jp/alignment/server/>) using E-INS-i strategy. Parameter of scoring matrix for nucleotide sequences

was set to 20PAM/ k = 2 as recommended when aligning related DNA sequences (Katoh & Toh, 2008).

ITS1 and *cox1* genetic diversity in *M. yucatanica* and in other available *Melipona* sequences were measured in DNAsp (Librado & Rozas, 2009), GenAlex (Peakall & Smouse, 2012) and MEGA 5 (Tamura et al., 2011) using various variability indices: number of genotypes, genotype diversity, variable sites, parsimony-informative sites, nucleotide diversity, and intraspecific genetic distance (K2P).

#### Phylogenetic analysis

The best-fit evolutionary model for the Bayesian analyses was determined with jModeltest 0.1.1 (Posada, 2008) using the Akaike information criteria (AIC). Bayesian analyses were carried out using MrBayes version 3.1 (Ronquist & Huelsenbeck, 2003). Separate runs were carried out with four simultaneous Markov chains, each starting from a random tree. The analysis ran for 6 500 000 generations to allow runs to converge, and the chain was sampled every 100th generation. The first 14 000 trees were discarded as the “burn-in” before the chains converged on a stable value and the posterior probabilities of tree topology were determined from the remaining trees. Also convergence was reassessed using the potential scale reduction factor (PSRF, Ronquist & Huelsenbeck, 2003).

## Results

#### ITS1 and *cox1* sequence variation

The two *M. yucatanica* worker bees analyzed per colony showed the same ITS1 sequence, therefore only one sequence per colony was used in the subsequent analyses. The first 126 base pairs (bp) of the alignment correspond to the

**Table 2.** Molecular diversity of the ITS1 fragments (complete and partial 3' and fragment) and cox 1 (3' fragment) in five *Melipona* species. Data from *M. yucatanica* in bold. Data from other *Melipona* sp. obtained from GenBank.

Dataset	ITS1 complete (1671 bp)						ITS1 partial (694 bp)						cox1 (580 bp)											
	bp	N	h	Gd	Pi	V	Par	d	bp	N	h	Hd	Pi	V	Par	d								
<i>Melipona yucatanica</i>	<b>1468-1472</b>	<b>11</b>	<b>3</b>	<b>0.250</b>	<b>0.0028</b>	<b>8</b>	<b>8</b>	<b>0.3</b>	<b>595-601</b>	<b>11</b>	<b>2</b>	<b>0.509</b>	<b>0.0009</b>	<b>1</b>	<b>1</b>	<b>0.086</b>	<b>580</b>	<b>7</b>	<b>4</b>	<b>0.81</b>	<b>0.0136</b>	<b>34</b>	<b>14</b>	<b>1.39</b>
<i>Melipona beecheii</i>	1519-1582	10	9	0.978	0.01239	40	20	1.21	555-558	10	5	0.844	0.01182	6	4	1.04	580	46	18	0.913	0.00744	25	16	0.75
<i>Melipona quinquefasciata</i>	-	-	-	-	-	-	-	-	570-609	9	9	1.00	0.0181	65	10	2.97	-	-	-	-	-	-	-	-
<i>Melipona subnitida</i>	-	-	-	-	-	-	-	-	590-612	13	13	1.00	0.06018	185	44	7.04	-	-	-	-	-	-	-	-
<i>Melipona quadrifasciata</i>	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	580	145	31	0.876	0.00509	55	32	0.51

bp = length of the fragment in base pairs, N = number of individuals, h = number of genotypes/haplotypes, Gd: genotype diversity, Hd: haplotype diversity, Pi = nucleotide diversity, V = variable sites, Par = parsimony-informative sites, d = intraspecific genetic distance with K2P (in %).

18S ribosomal region and the last 58 bp to the 5.8S, so the size of the ITS1 region was 1310-1312 bp in Guatemalan and 1308 bp in Mexican *M. yucatanica*. Insertion-deletion (indel) events were observed in the alignment of the ITS1 sequence of *M. yucatanica* specimens from Guatemala and México. Two indels of 4 and 8 bp corresponded to a microsatellite locus showing different number of repeat motifs. This variation in the number of motifs was not detected among the specimens within each population but between them. An additional variation was detected among Guatemalan *M. yucatanica* specimens, due to the presence of one insertion of two base pairs (TT) in the specimen 4 (*MyucGuat4*). In relation to other species, a large insertion of 80 bp was detected in *M. quadrifasciata*.

There were three genotypes, one for Mexican (n = 7, *MyucMer1-7*) and two for Guatemalan individuals (n=1; *MyucGuat4* and n = 3 *MyucGuat1*, 2 and 5). A higher number of unique genotypes was found in *M. beecheii*, *M. quinquefasciata* and *M. subnitida* populations (Table 2).

The ITS1 nucleotide diversity (Pi) was from 3 to 65 times lower for *M. yucatanica* than for the other three *Melipona* species (Table 2). This result contrasts with the nucleotide diversity and the genetic distances (K2P) values obtained for cox1, where *M. yucatanica* population had similar values or slightly higher (1.8 to 2.7 times higher) than those obtained for other available species (Table 2).

Comparisons between markers showed that in *M. yucatanica*, ITS1 nucleotide diversity (pi = 0.0028) and genetic distance (d = 0.28%) is about five times lower than in cox1 (pi = 0.0136, d = 1.39%). On the other hand, in *M. beecheii* the comparison of both markers showed a different pattern, as the diversity and distance values are about 1.6 times higher with the ITS1 than with cox1 data (Table 2).

### Phylogenetic analyses

The optimal model of nucleotide substitution, following AIC criterion, was the transversional model (TVM). Bayesian analysis of the complete ITS1 region, showed that this species is divided in two well supported clades (Mexican and Guatemalan specimens, p.p. = 1.0) defining a clear phylogeographic structure (Fig 2), *M. beecheii* showed two clades with the same geographic structure, whereas *M. subnitida* and *M. quinquefasciata* showed a more complex pattern of population structure. *M. yucatanica* ITS1 sequences clustered with high support (p.p = 1.0) within the subgenus *Melipona s. str.* together with *M. quadrifasciata* and *M. subnitida*.

Analysis of available cox1 sequences resulted in the same two well supported clades of Mexican and Guatemalan populations (Fig 3). Moreover the species *M. yucatanica* was also grouped with high support within the subgenus *Melipona s. str.* The combined analysis of cox1 and ITS1 resulted in the same well supported clades (Fig S1 in Supplementary Material).

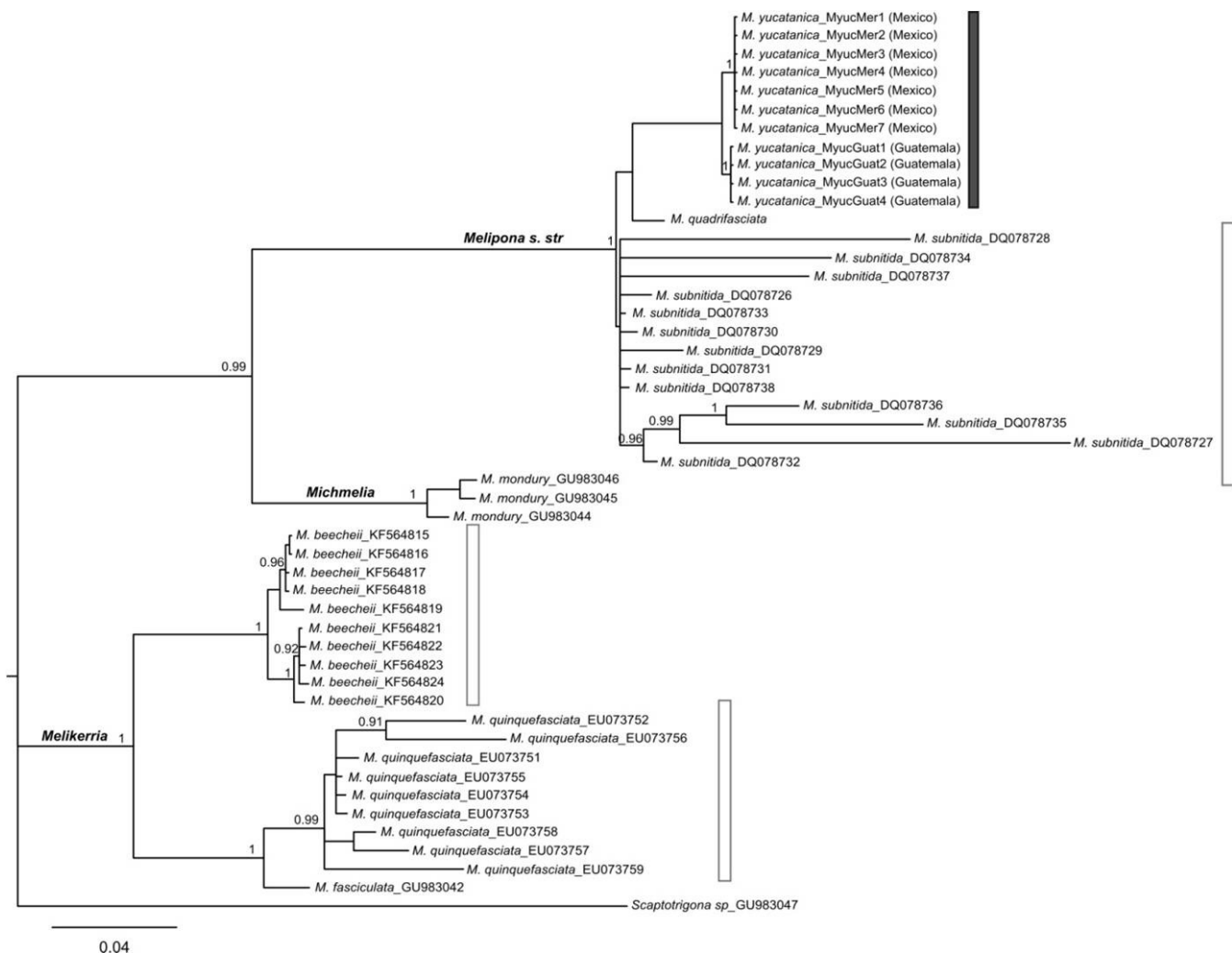
## Discussion

Phylogenetic analyses showed low genetic variation in ITS1 between populations but defined a geographic structure with Mexican and Guatemalan specimens forming two well supported clades. The characterization of the ITS1 region of the stingless bee *M. yucatanica* further evidenced that this region is longer in stingless bees than in other Hymenoptera (Pilgrim & Pitts, 2006; Taylor et al., 2006). In *M. yucatanica* ITS1 is within the range of that observed in other *Melipona* species (from 1391 bp in *M. quadrifasciata* to 1940 in *M. rufiventris*, Fernandes-Salomão et al., 2005). The presence of insertions and deletions (indels) in this region is a common feature leading to sequence length variation, as it has been observed between Mexican and Guatemalan *M. yucatanica* specimens, and among Brazilian populations of the species *M. subnitida* (Cruz et al., 2006) and within *M. quinquefasciata* (Pereira et al., 2009).

The ITS1 clades separate between Mexican and Guatemalan populations showing the same phylogeographic pattern found in previous markers (ITS2, De la Rúa et al.,

2007; *cox1* and microsatellites May-Itzá et al., 2010). These results are congruent with the fact that both populations are located in opposite extremes of the species distribution range (Ayala, 1999). However, an incongruent pattern of genetic variability was revealed when several markers were compared. Populations of *M. yucatanica* showed lower levels of genetic diversity and genetic distance in the ITS1 region than those reported for other markers as *cox1* and microsatellite data (May-Itzá et al., 2010; Table 2). ITS1 and *cox1* have different mechanisms of evolution that have led to different patterns in intraspecific variations for various taxa (e.g., Hansen et al., 2006; Carlini et al., 2009; Kornobis & Pálsson, 2011). The low variation of ITS1 could therefore be explained by intraspecific rDNA ITS1 homogenization due to the mechanisms of concerted evolution (Hillis & Dixon, 1991).

However, this explanation cannot be extended to other *Melipona* species. In *M. beecheii*, a species with wider geographic distribution, the opposite pattern was found with diversity and distance values of ITS1 higher than for *cox1* data (May-Itzá et al., 2010). Moreover, when data are

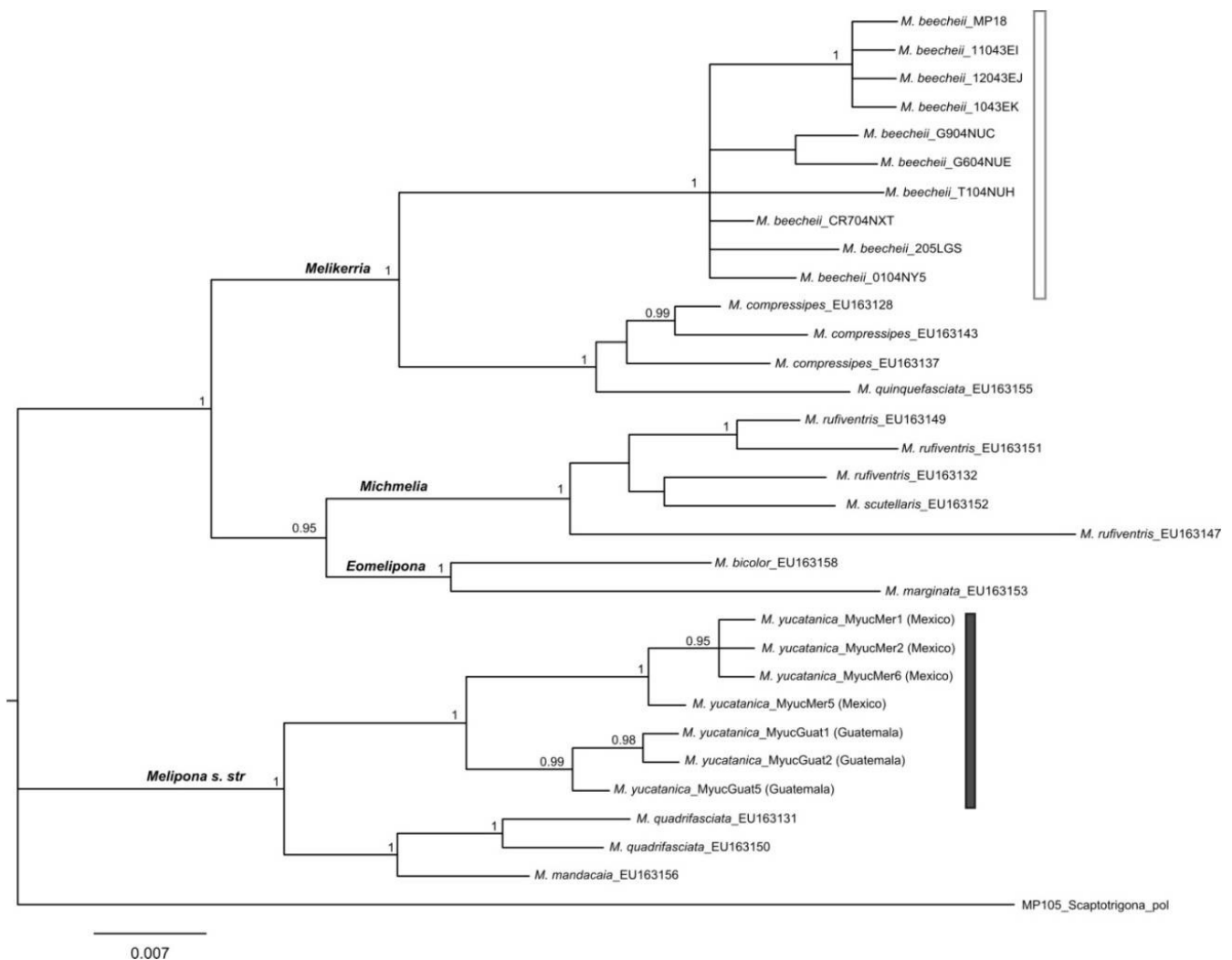


**Fig 2.** Bayesian analyses of the ITS1-complete region of several *Melipona* species. Population data of *Melipona yucatanica* are indicated with a solid bar. Available population data of several *Melipona* species are indicated with empty bars. Numbers above nodes show posterior probability  $\geq 0.90$ .

compared between these two species, *M. beecheii* showed about four times higher divergence, genotype and nucleotide diversity of ITS1 (complete fragment) but almost two times lower values for *cox1* data (Table 2). Unfortunately, there are no available data to compare this pattern with other *Melipona* species; however, *M. yucatanica* showed a remarkably lower ITS1 divergence (from 12 to 80 times) and higher *cox1* divergence (from 1.8 to 2.7 times) than all other *Melipona* species analyzed (Table 2). This observed distinct pattern of divergence between nuclear and mitochondrial markers in *M. yucatanica* populations in relation to other *Melipona* species could be due to several causes which will require specific testing, among them: i) different efficiency of concerted evolution of ITS1 rDNA between *Melipona* species, as suggested for other taxa (Armbruster & Korte, 2006); ii) a complete replacement in one of the sampled populations of the original mitochondrial haplotype by introgression which led to higher levels of mitochondrial divergences. This hypothesis cannot be disregarded although until date, introgression

events have not been described in *Melipona* species yet; and iii) distinct population dynamics in relation to other the *Melipona* species analyzed. Further analyses including more markers and more *M. yucatanica* populations scattered across its distribution range are needed in order to understand the population dynamic of this endangered species.

The phylogenetic signal obtained in the ITS1 Bayesian analysis confirms the utility of this region as a potential phylogenetic marker for the genus *Melipona*. This marker seems suitable for resolving population and subgenera divergences. In this sense, the phylogenetic analysis included *M. yucatanica* in the subgenus *Melipona s. str.* together with other species as *M. quadrifasciata* and *M. mandacaia*. This molecular result confirms previous morphological studies (Moure et al., 2007) and it is in accordance with recent molecular studies of the genus *Melipona* (Ramírez et al. 2010). In the light of recent numt DNA descriptions in other *Melipona* subspecies (Cristiano et al., 2012; Ruiz et al., 2013), the ITS1 region has demonstrated its potential as an appropriate marker for genetic studies in stingless bees.



**Fig 3.** Bayesian analysis of the available *cox1* gene from *Melipona* species. Numbers above nodes show posterior probability  $\geq 0.90$ . Population data of *Melipona yucatanica* are indicated with a solid bar. Available population data of several *Melipona* species are indicated with empty bars.

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## References

- Armbruster, G.F.J. & Korte, A. (2006). Genomic nucleotide variation in the ITS1 rDNA spacer of land snails. *J. Moll. Stud.*, 72: 211–219.
- Arias, M.C., Brito, R.M., Francisco, F.O., Moretto, G., de Oliveira, F.F., Silvestre, D. & Sheppard, W.S. (2006). Molecular markers as a tool for population and evolutionary studies of stingless bees. *Apidologie*, 37: 259–274. DOI: 10.1051/apido:2006021
- Ayala, R. (1999). Revisión de las abejas sin aguijón de México (Hymenoptera: Apidae: Meliponini). *Folia Entomol. Mex.*, 106:1 - 123.
- Camargo, J.M.F., Moure, J.S. & Roubik, D.W. (1988). *Melipona yucatanica* New Species (Hymenoptera: Apidae: Meliponinae): stingless bee dispersal across the Caribbean Arc and Post-Eocene Vicariance. *Pan-Pacific Entomol.*, 64: 147–157.
- Camargo, J.M.F. & Pedro, S.R.M. (2007). Meliponini Lepeletier, 1836. In Moure, J.S., Urban, D. & Melo, G.A.R. (Eds). *Catalogue of Bees (Hymenoptera, Apoidea) in the Neotropical Region* - online version. Available at <http://www.moure.cria.org.br/catalogue>. (Accessed Oct/19/2010).
- Carlini, D.B., Manning, J., Sullivan, P.G. & Fong, D.W. (2009). Molecular genetic variation and population structure in morphologically differentiated cave and surface populations of the freshwater amphipod *Gammarus minus*. *Mol. Ecol.*, 18: 1932–1945. DOI: 10.1111/j.1365-294X.2009.04161.x
- Cristiano, M.P., Fernandes-Salomão, T.M. & Yotoko, K.S.C. (2012). Nuclear mitochondrial DNA: an Achilles' heel of molecular systematics, phylogenetics, and phylogeographic studies of stingless bees. *Apidologie*, 43: 527-538. DOI: 10.1007/s13592-012-0122-4.
- Cruz, D.O., Jorge, D.M.M., Pereira, J.O.P., Torres, D.C., Soares, C.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. DOI: 10.1051/apido:2006003
- 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. DOI: 10.1007/s00040-007-0962-5
- 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. DOI: 10.1007/s00040-004-0767-8
- Hansen, H., Martinsen, L., Bakke, T.A. & Bachmann, L. (2006). The incongruence of nuclear and mitochondrial DNA variation supports conspecificity of the monogenean parasites *Gyrodactylus salaris* and *G. thymalli*. *Parasitology*, 133: 639–650 DOI: 10.1017/S0031182006000655
- Hillis, D.M. & Dixon, M.T. (1991). Ribosomal DNA: molecular evolution and phylogenetic inference. *Q. Rev. Biol.*, 66: 411–453.
- 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. DOI: 10.1046/j.1471-8286.2003.00519.x
- Katoh, K. & Toh, H. (2008). Improved accuracy of multiple ncRNA alignment by incorporating structural information into a MAFFT-based framework. *BMC Bioinformatics*, 9: 212. DOI: 10.1186/1471-2105-9-212
- Kornobis, E. & Pálsson, S. (2011). Discordance in Variation of the ITS Region and the Mitochondrial COI Gene in the Subterranean Amphipod *Crangonyx islandicus*. *J. Mol. Evol.*, 73: 34-44. DOI: 10.1007/s00239-011-9455-2
- Librado, P. & Rozas, J. (2009). DnaSP v5: A software for comprehensive analysis of DNA polymorphism data. *Bioinformatics*, 25: 1451-1452. DOI: 10.1093/bioinformatics/btp187
- May-Itzá, W. de J., Quezada-Euán, J.J.G. & De la Rúa, P. (2009). Intraspecific variation in the stingless bee *Melipona beecheii* assessed with PCR-RFLP of the ITS1 ribosomal DNA. *Apidologie*, 40: 549–555. DOI: 10.1051/apido/2009036
- May-Itzá, W. de J., Quezada-Euán, J.J.G., Medina Medina, L., Enríquez, E. & De la Rúa, P. (2010). Morphometric and genetic differentiation in isolated populations of the endangered Mesoamerican stingless bee *Melipona yucatanica* (Hymenoptera: Apoidea) suggest the existence of a two species complex. *Conserv. Genet.*, 11: 2079-2084. DOI: 10.1007/s10592-010-0087-7
- May-Itzá, W. de J., Quezada-Euán, J.J.G., Ayala, R., & De la Rúa, P. (2012). Morphometric and genetic analyses reveal two taxonomic units within *Melipona beecheii* (Hymenoptera: Meliponidae), a Mesoamerican endangered stingless bee. *J. Insect. Conserv.*, 16: 723–731 DOI: 10.1007/s10841-012-9457-4
- Moure, J.S., Urban, D. & Melo, G.A.R. (2007). *Catalogue of Bees (Hymenoptera, Apoidea) in the Neotropical Region*.

Curitiba: Sociedade Brasileira de Entomologia, 1058 p

Peakall, R. & Smouse, P.E. (2012). GenAlEx 6.5: genetic analysis in Excel. Population genetic software for teaching and research-an update. *Bioinformatics*, 28: 2537-2539. DOI: 10.1093/bioinformatics/bts460

Pereira, J.O.P., Freitas, B.M., Jorge, D.M.M., Torres, D.C., Soares, C.E.A. & Grangeiro, T.B. (2009). Genetic variability in *Melipona quinquefasciata* (Hymenoptera, Apidae, Meliponini) from northeastern Brazil determined using the first internal transcribed spacer (ITS1). *Gen. Mol. Res.*, 8: 641-648.

Pilgrim, E.M. & Pitts, J.P. (2006). A molecular method for associating the dimorphic sexes of velvet ants (Hymenoptera: Mutillidae). *J. Kans. Entomol. Soc.*, 79: 222-230.

Posada, D. (2008). jModelTest: Phylogenetic Model Averaging. *Mol. Biol. Evol.*, 25: 1253-1256. DOI:10.1093/molbev/msn083

Ramírez, S.R., Nieh, J.C., Quental, T.B., Roubik, D.W., Imperatriz-Fonseca, V.L. & Pierce, N.E. (2010). A molecular phylogeny of the

stingless bee genus *Melipona* (Hymenoptera: Apidae). *Mol. Phyl. Evol.*, 56: 519-525. DOI: 10.1111/j.1365-3113.2006.00362.x

Ronquist, F. & Huelsenbeck, J.P. (2003). MrBayes 3: Bayesian phylogenetic inference under mixed models. *Bioinformatics*, 19: 1572-1574. DOI:10.1093/bioinformatics/btg180

Ruiz, C., May-Itzá, W. de J., Quezada-Euán, J.J.G. & De la Rúa, P. (2013). Nuclear copies of mitochondrial fragments (numts) and phylogenetic analysis of two related species of stingless bee genus *Melipona* (Hymenoptera: Meliponini). *J. Zool. Sys. Evol. Res.*, 51: 107-113. DOI: 10.1111/jzs.12011

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M., & Kumar, S. (2011). MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol. Biol. Evol.*, 28: 2731-2739. DOI:10.1093/molbev/msr121

Taylor, D.B., Moon, R., Gibson, G. & Szalanski, A. (2006). Genetic and Morphological Comparisons of new and old world populations of *Spalangia* species (Hymenoptera: Pteromalidae). *Ann. Entomol. Soc. Am.*, 99: 799-808. DOI: 0.1603/0013-8746(2006)99[799:GAMCON]2.0.CO;2

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