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Genetic Variability of *Melipona subnitida* (Hymenoptera: Apidae) in Introduced and Native Populations

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Abstract

Melipona subnitida (Hymenoptera: Apidae) is a stingless bee native to Caatinga biome in Brazil, well adapted to hot and dry climate of that region and has been traditionally explored for honey production. Here, we evaluate the genetic structure of 173 colonies of *M. subnitida* in northeast Brazil by partially sequenced mitochondrial genes cytochrome oxidase I (COI) to compare an introduced population isolated for 30 yr into the Island of Fernando de Noronha (IFN) with the continental populations. We identified high haplotype diversity (0.8220) with 14 haplotypes on the continental populations, being three new ones, compared with the database GenBank. The haplotype H4 was present at the center of network, occurring in four localities on mainland and fixed as a single haplotype on IFN. We propose that the island populations originally introduced carried one haplotype (H4), even though IFN population is suffering pressure by island effect through changes on morphology. Studies on island populations could be a model to understand the dynamics of isolated populations and sustainable management of this biome to preserve *M. subnitida*.

Key words: genetic diversity, island, cytochrome oxidase I gene, mitochondrial DNA, stingless-bee

The introduction of species into an environment is a threat to biological diversity and may cause economical losses (Oliveira 2004, Vitule and Prodocimo 2012). Among the consequences, species competition, local extinction, introduction of plagues and diseases, and loss of genetic variability are more likely to occur (Delariva and Agostinho 1999).

Despite this, the introduction of bee's species is often associated with a positive impact due to their pollination services (Russo 2016). As, per example, the introduction of stingless bees and *Apis mellifera* L. (Hymenoptera: Apidae) carried out on the oceanic Island of Fernando de Noronha (IFN), 345 km off the Brazilian coast, in order to increase the pollinating services, since the local bee fauna was poor (Kerr and Cabeda 1985).

In 1983, 30 colonies of 'jandaíra' (*Melipona subnitida* Ducke, 1910 (Hymenoptera: Apidae)) a stingless bee native and endemic to the Caatinga biome in northeast Brazil (Camargo and Pedro 2007) was introduced outside its natural distribution, on IFN. Originally, the islands colonies were from continent, Fortaleza (Ceará State, 20 colonies) and Mossoró (Rio Grande do Norte State, 10 colonies) (Kerr and Cabeda 1985).

Islands are an example of naturally fragmented areas (Boff et al. 2014), being characterized by low genetic diversity due to bottlenecks, founder effects and genetic drift, which implicates that genetic factors play an important role on islands populations and the process to colonizing new areas (Matute 2013).

On mainland, understanding the process of genetic diversity, population structure, dispersal capacity, and gene flow of species on the world biota, in particular, the Caatinga biome, is fundamental to genetic conservation. Above all, Caatinga is the principal biome in the northeastern region and one of the most threatened environments

of the planet. Its losses are of 45% (826,400 km²) of its original territory mainly due to deforestation, logging and replacement of native species for pastures and crops (Brasil 2015). Nevertheless, the Caatinga covers more than 750,000 km², with a large number of species adapted to annual high temperatures and extremely irregular rainfall rates over the years, resulting in periodic severe droughts (Leal et al. 2003, Oliveira et al. 2012).

This study aimed to evaluate, through mitochondrial genetic markers, the genetic diversity of *M. subnitida* bees introduced at the island and, consequently, the effects of genetic drift, natural selection and inbreeding for a period of 30 yr, compared to populations of the same species in their natural area of distribution in northeast Brazil.

Materials and Methods

Samples

To assess the genetic diversity of *M. subnitida* across North Eastern Brazil, partial sequences of mitochondrial DNA COI were used through sequences obtained from 173 managed bee workers. These bees belonged to 13 colonies collected at the island of Fernando de Noronha in 2013, and 160 colonies-spread in continental areas: 1) colonies in the states of Bahia (n = 13), Alagoas (n = 23), and Pernambuco (n = 32) (access numbers in the GenBank: KT378608 to KT378612) and 2) colonies in the states of Rio Grande do Norte (n = 66), Maranhão (n = 7), Piauí (n = 9) and Ceará (n = 10), from Bonatti et al. (2014) (access numbers in the GenBank: KC 879031.1 to KC 879041.1) (Table 1 and Fig. 1).

Mitochondrial DNA Analysis

Total DNA was extracted from head tissue of each bee worker using Wizard Genomic DNA Purification Kit (Promega). The amplification of the COI region of the mitochondrial DNA (mtDNA) used *primers* mtD6 and mtD9 (Simon et al. 1994). The PCR reactions of 10 µl containing 5 µl of Top Taq Master Mix (Qiagen), 0.2 µl of each primer at 20 mm, 4.1 µl of ultra-pure water and 0.5 µl of total DNA. The PCR used the following program: denaturation for 5 min at 94°C, 35 cycles of 60 s at 94°C, 60 s at 42°C, 3 min at 64°C, and a final extension cycle at 64°C for 10 min. The amplified fragments were visualized on 2% agarose gels stained with Gel-Red (Biotium). The products

of COI gene amplification were purified with the DNA precipitation protocol with PEG 8000 (polyethylene glycol) at 20%. Sequencing reactions were conducted by the direct method, *forward and reverse*, containing: 50 ng of purified PCR product, 1.75 µl of sequencing buffer, 0.5 µl of BigDye v3.1 (Applied Biosystems), 0.25 µl of oligonucleotide (pmol/µl) starter and ultrapure water to complete 10 µl. The sequencing reaction consisted of 1 cycle of 96°C for 3 min, 35 cycles consisting of a phase at 96°C for 15 s, a phase at 42°C for 10 s and a polymerization phase at 60°C for 4 min, and finally, a phase at 60°C for 5 min. The samples were then kept at 4°C until used.

Afterward, 40 µl of isopropanol 80% was added to each sample. After 15 min at room temperature, the samples were centrifuged at 4000 rpm, in a refrigerated centrifuge for 25 min at 4°C. After DNA precipitation, the supernatant was discarded and the samples were washed twice with 150 µl of ethanol 70%, followed by centrifugation at 4000 rpm for 15 min at 4°C. The samples were dried at room temperature or in thermal cycler (50°C for 15 min), resuspended in 10 µl of formamide, denatured for 5 min at 95°C, and submitted to sequencing on an automatic sequencer 3130XL DNA Analyzer (Applied Biosystems).

Sequence Analysis

The alignment of the sequences was edited in the program BioEdit (Hall 2005). The sequences were aligned through the tool MUSCLE (Edgar 2004). The multiple alignment file was analyzed with the MEGA v6.0 software (Tamura et al. 2013). DNA sequences were conducted using the DnaSP program v.5.1 (Librado and Rozas 2009), which estimated the number of mitochondrial haplotypes (h), haplotype diversity (Hd), nucleotide diversity (π), number of polymorphic sites (S), and average number of nucleotide differences (k).

The analysis of molecular variance (AMOVA) (Excoffier et al. 1992) was implemented by the program Arlequin 3.5 (Excoffier and Lischer 2010), in order to check the differences between populations, and the Fst index, between pairs of populations. The intrapopulation diversity and interpopulation divergence were calculated using the model of differentiation pair-by-pair. Inferences in respect to the occurrence of population expansion were based on estimates of neutrality using the D tests of Tajima (1989) and Fs tests of Fu (1997).

Table 1. Geographic location of samples of *Melipona subnitida*

State	Location	ID map	N colonies	S	Hd	π	K
Pernambuco	Island of Fernando de Noronha	1	13	0	0.000	0.000	0.000
	Cumaru	2	7	0	0.000	0.000	0.000
	Exu	3	3	2	0.667	0.00267	1.333
	Passira	4	5	0	0.000	0.000	0.000
	Riacho das Almas	5	6	2	0.333	0.00133	0.667
	Taquaritinga do Norte	6	11	1	0.327	0.00065	0.327
Alagoas	Água Branca	7	11	2	0.345	0.00073	0.364
	Mata Grande	8	12	1	0.303	0.00061	0.303
Bahia	Joá	9	5	1	0.600	0.00120	0.600
	São José	10	8	1	0.536	0.00107	0.536
Rio Grande do Norte	Areia Branca	11	6	2	0.533	0.00239	1.067
	Jandaíra	12	49	7	0.690	0.00473	2.109
	Mossoró	13	11	2	0.436	0.00171	0.873
Maranhão	Barreirinhas	14	7	1	0.571	0.00128	0.571
Piauí	Parnaíba	15	9	3	0.806	0.00249	1.111
Ceará	Fortaleza	16	10	2	0.511	0.00125	0.556
Total			173	13	0.8220	0.00391	1.706

Contents of genetic diversity of the COI gene. (S) number of polymorphic sites; (Hd) haplotype diversity; (π) nucleotide diversity; (K) average number of differences.
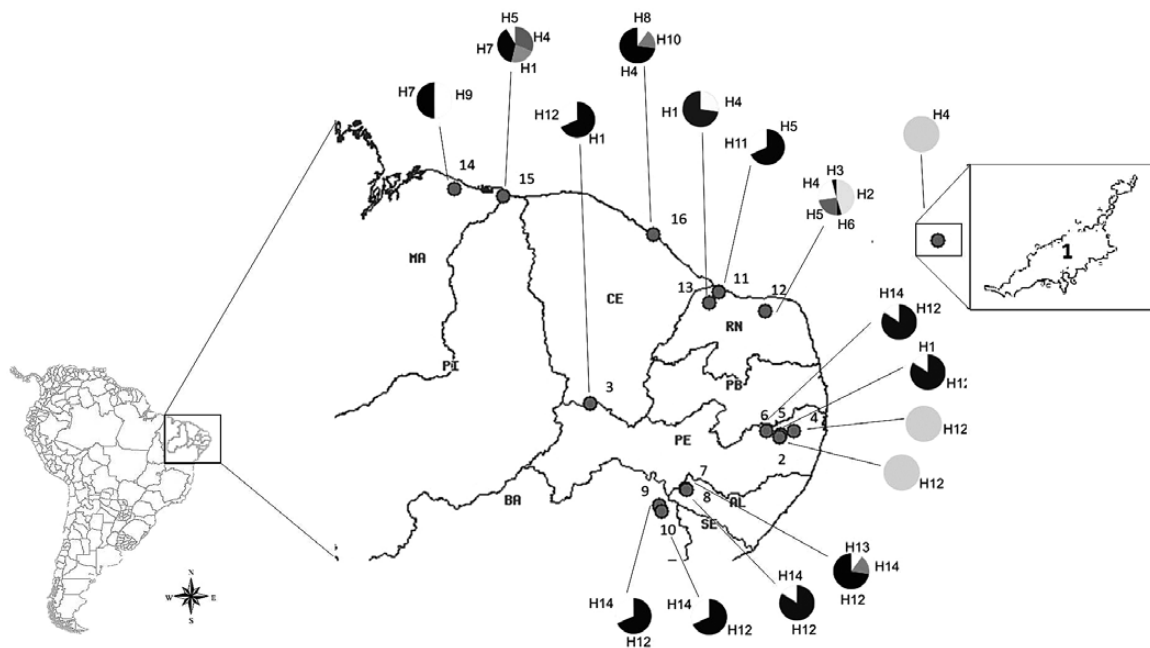
Fig. 1. Map of geographic location and distribution of the mitochondrial sequences of *Melipona subnitida*. The numbers refer to the location codes shown in Table 1.

To estimate the genealogical relationships between mitochondrial haplotypes, a network of haplotypes was built using NETWORK v.4613 (www.fluxus-engineering.com) generated from the median-joining method (Bandelt et al. 1999). The size of each group refers to the haplotype frequency and filling at different localities.

Results

The alignment was set at 510 bp, with 497 sites preserved (monomorphic) and 13 variable sites (polymorphic) with just a single site (singleton or point mutation). The COI gene presented a nucleotide frequency average of $T = 32.5\%$, $A = 44.5\%$, $C = 9.6\%$, and $G = 13.3\%$. We recorded 39 substitutions, being 37 transitions, and 2 transversions.

Hd was 0.8220. However, the continental samples of Cumaru and Passira and IFN showed only one haplotype. The highest diversity 0.806 was obtained in Parnaíba, with four haplotypes. The index of nucleotide diversity (π) corresponded to an average of 0.00391, without variations in populations of IFN, Cumaru and Passira, in contrast to 0.00473 in Jandaíra (Table 1). Considering only continental populations (excluding the colonies introduced on the island), the haplotype and nucleotide diversity indices remained high (Hd = 0.824; π = 0.00410; k = 1.786; n = 160).

We identified 14 haplotypes (H1 to H14), which differ from 1 to 6 substitutions in the nucleotide bases (Supp. Table S1). Of these, 11 (H1 to H11) had already been deposited at the NCBI database and three (H12, H13, and H14) are new haplotypes. In addition, there was an expansion of fragments of the sequences of haplotypes H1 and H4. Haplotype H12 was the most frequent, occurring in nine populations of the southern border and H1 is the only haplotype found in borders of the natural distribution of the species (North and South).

The analysis of haplotypes network (Fig. 2) suggests the existence of four main groups (H1, H2, H4, and H12). The first, formed only by H1; the second, composed of H2, H3, and H6, restricted to Jandaíra (RN); the third group, with H4 more basal, positioned in the center and with haplotypes H5, H7, H8, H10, and H11 distant from H4 by a single mutational step; and the fourth group, consisting of the haplotypes H12, H13, and H14, unique in the southern border of the natural distribution of the species.

Only the haplotype H4 was found in IFN. H4 also is present in four localities in the North, including Mossoró and Fortaleza, original sites of the colonies of *M. subnitida* introduced on the island in 1983.

The intra- and inter-populational variations were highly significant (Table 2). For the tests of selective neutrality of mutations, the D test of Tajima presented a mean value of 0.18780 and the Fs test a value of 0.52204, which were not statistically significant ($P > 0.05$). Nucleotide diversity reflected in the amino acid sequence with eight silent mutations (synonymous) and five non-synonymous mutations (Supp. Table S2).

The Φ_{ST} values was 0.43164 ($P < 0.00$; Table 2) and the pairwise Φ_{ST} (Supp. Table S3) show that the highest level of differentiation occurred among populations with a smaller number of haplotypes, similar to IFN and Cumaru and between IFN and Passira.

Discussion

The genetic diversity was higher in the mainland with 14 haplotypes and lower on the island. Furthermore, after 30 yr of isolation on IFN, *M. subnitida* populations fixed a single haplotype (H4), only present at the northern border of its natural distribution on mainland. Moreover, its presence at the center of network suggests that it is the most ancient, corroborating with Bonatti et al. (2014).

We addressed two hypotheses for the IFN colonization: 1) the colonies introduced in the island carried only H4 or 2) even though more haplotypes were introduced, environmental changes and stochastic factors resulted in the establishment of only one haplotype. Although H4 and other three haplotypes can be found on original colonies' sites (Fortaleza and Mossoró), which could corroborate hypostases II, there is no historical evidence to prove the existence of these haplotypes during bees' introduction on IFN. Moreover,
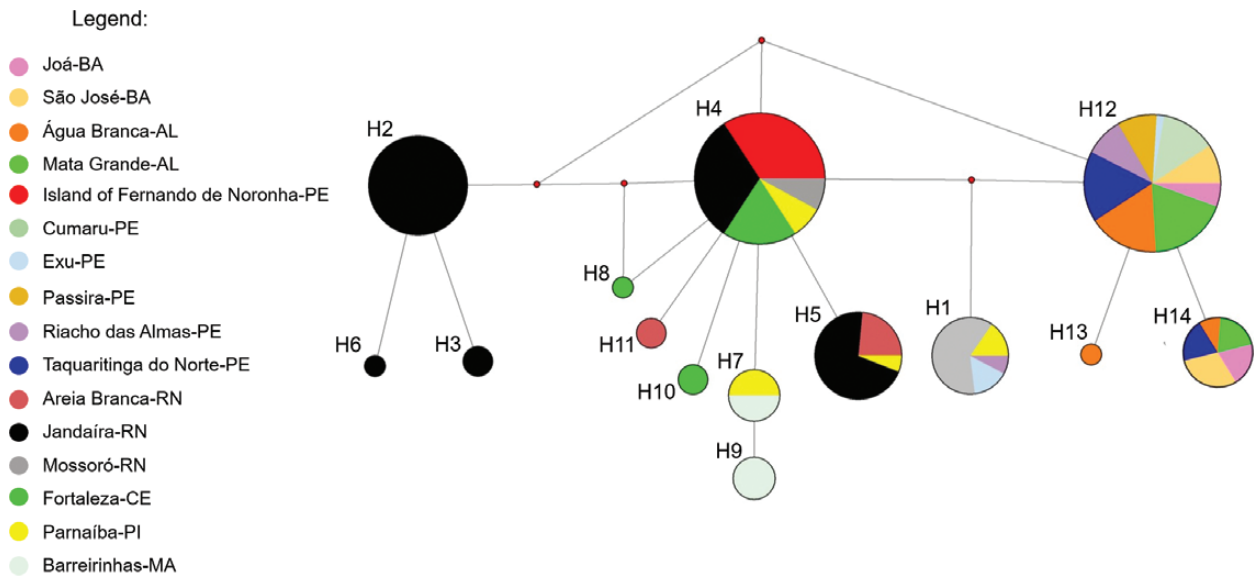
Fig. 2. Network of haplotype interrelations for the COI gene of *Melipona subnitida.* The sizes of the circles are proportional to haplotype frequency.

Table 2. Analysis of molecular variance (AMOVA) for sequences COI gene of *Melipona subnitida*

Structure	Variation	Degrees of freedom	Variance of components	% of variation	Φ	*P*-value
Populations from the island vs. populations from the continent	Intergroup	1	−0.06753	−8.00	$\Phi_{CT} = -0.08000$	0.49485
	Interpopulation	14	0.43185	51.16	$\Phi_{SC} = 0.47375$	0.00000
	Intrapopulation	157	0.47972	56.84	$\Phi_{ST} = 0.43164 \pm 0.00499$	0.00000
	Total	172	0.84404			

Number of changes = 10,100; α = 0.05.

the presence of H4 and the other haplotypes in mainland colonies could be the result of recent migration and genetic diversity loss on the island. Hence, by the parsimony, we propose that the originally introduced island's populations carried only one haplotype (the first hypothesis). Even though the IFN population is suffering pressure by island effect, as observed by changes on their morphology; their head is smaller than the ones from mainland bees (Souza et al. 2018).

Only few stingless bees have been studied concerning flight range and flight ability. Although they can fly large distances (Kuhn-Neto et al. 2009, Rodrigues and Ribeiro 2014) it is supposed that, in general, they fly short distances when searching for food resources, especially when they have small body size (Van Nieuwstadt and Iraheta 1996, Araújo et al. 2004). The same is observed for nesting behavior, as the new colony keeps communication with the mother nest for a period of time (Roubik 2006). In this way, stingless bees usually have a relatively low dispersion when swarming for reproduction.

Despite the lack of management of colonies, absence of feral bees and harsh conditions, such as prolonged drought periods (Ribeiro and Lima 2015), *M. subnitida* had reproductive success on the island. Even though is quite difficult to evaluate generations since stingless bees' colonies are perennial, the queen may live for 4–5 yr and the workers (her daughters) survive only a few months. The stingless bees' queen mates only once and mostly with a single male (Peters et al. 1999, Strassmann 2001), meaning only one generation exists for each queen and if this were the case in the island, only six generations passed in the last 30 yr.

Alves et al. (2011) had already reported the success in maintaining colonies of *Melipona scutellaris* Latreille (Hymenoptera: Apidae)

for 10 yr, despite the severe genetic bottleneck effect. Regarding conservation efforts of this endemic species of the northeastern region of Brazil, it should be taken into consideration that inbreeding and genetic drift may have less severe consequences to *M. subnitida* or at least to haplotype H4 remaining in this population, even though loss of genetic variability can happen, particularly for nuclear DNA including CSD locus.

On the mainland, we identified three new haplotypes (H12, H13, and H14). They make up a group restricted to the southern border of the species natural distribution, probably due to geographical barriers. These barriers may be the altitude due to natural occurring elevations, as Chapada do Araripe (states of Ceará, Piauí, and Pernambuco) and due to the environmental degradation of the Caatinga biome, which prevents or hinds the occurrence of genetic flow (Potts et al. 2016, Giannini et al. 2017).

This fact, accompanied by other such as environmental degradation, lack of nesting sites and/or food resources, competition with other species, and diseases could explain the low expansion of the *M. subnitida* populations on mainland by neutrality test. Regardless, *M. subnitida* is highly established on the sites sampled, based on Φ_{ST} value, there is little or no migration occurring additionally the fact that the allele frequencies within each population are different. Other stingless bees also showed higher Φ_{ST}, as *Melipona quadrifasciata* Lepeletier (Hymenoptera: Apidae) with 0.59 (Nascimento et al. 2010) and 0.90 (Batalha-Filho et al. 2010), *Melipona rufiventris* Lepeletier (Hymenoptera: Apidae) with 0.76 and 0.77 (Tavares et al. 2007), and moderately structured in *Melipona mandacaia* Smith (Hymenoptera: Apidae) with 0.2961 (Miranda et al. 2012).

High intrapopulation genetic variability is generally associated with the lack or the restrictions on genetic flow between colonies of different localities, which can potentially lead to an increase of inbreeding within localities (Miranda et al. 2012). Within these settings, most populations are genetically diverse, containing different haplotypes restricted to them. Conversely, some populations of *M. subnitida* present only one haplotype contrasting the high diversity found on mainland.

Our study has important implications for conservation of native bee species, as a model for understanding the dynamics of isolated populations in *M. subnitida* on fragmented landscapes, since this already occur in natural areas. We stress the importance of conservation and sustainable management of this biome and the preservation and maintenance of the species. In future research, accessing-genetic diversity by nuclear DNA could provide the answers that are currently required to understand the island effect occurring on the IFN population.

Supplementary Data

Supplementary data are available at *Journal of Insect Science* online.

Acknowledgments

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