Sociobiology 65(4): 645-653 (October, 2018) Special Issue DOI: 10.13102/sociobiology.v65i4.3464

Sociobiology
An international journal on social insects

RESEARCH ARTICLE - BEES

Two Colors, One Species: The Case of *Melissodes nigroaenea* (Apidae: Eucerini), an Important Pollinator of Cotton Fields in Brazil

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Article History

Edited by
Eduardo Almeida, USP, Brazil
Received 10 May 2018
Initial acceptance 02 July 2018
Final acceptance 26 August 2018
Publication date 11 October 2018

Keywords
Cytochrome oxidase I, Bayesian phylogeny, bees, barcoding gap.

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Abstract

Accurate taxonomic delimitation in ecological research is absolutely critical as studies that seek to evaluate levels of biodiversity and quantify human effects on the environment are rapidly undertaken. Coloration is a widely used morphological character for species identification through dichotomous keys. However, taxonomic identification based upon coloration is often unreliable because this character can exhibit high degree of intraspecific variation. An uncertain interpretation of mesosoma color-morphs (yellow or black) occurred when we used this character, in association with the bristle bands in the TII, for the identification of eucerine bee *Melissodes nigroaenea* (Smith), an important pollinator of cotton fields in South America. Thus, we used a DNA barcoding approach to investigate if different color morphs within this species reflected two distinct evolutionary lineages. Our Bayesian phylogenetic reconstruction revealed that both yellow and black individuals clustered in a highly supported monophyletic group. Additionally, pairwise genetic distances between *M. nigroaenea* color-morphotypes were lower than 3%. These results indicate that both mesosoma color-morphs correspond to intraspecific variability within the same evolutionary unit. Although the mesosoma coloration is not the unique character considered for taxonomic differentiation among *Melissodes* species, our results indicate that this character is not reliable for *Melissodes* species differentiation. The incorporation of DNA barcoding approaches to taxonomic classification can help resolve some of the problems that originate while relying on purely morphological taxonomy.

Introduction

Accurate species identification is crucial for biodiversity studies that aim to characterize patterns of species richness and abundance, and to identify recent changes in these patterns as a result of anthropogenic activities (Austen et al., 2016; Packer et al., 2018). A critical step for biodiversity studies is the reliable identification of collected individuals to the species level. In some cases, independent evolutionary units with very similar morphological characters might be classified as one species leading to an underestimation of biological diversity (Vodă et al., 2015). Evolutionary lineages which have a recent divergence time but cannot be differentiated morphologically are the result of a process called cryptic speciation (Bickford et al., 2007). On the other hand, species may exhibit polymorphism, a phenomenon whereby conspecific individuals display marked variation in morphological characters. If these polymorphic characters are used for species identification, the same evolutionary unit may be assigned to different species, leading to an overestimation of biodiversity (Sigovini et al., 2016). When cryptic or polymorphic species are present in a community,

species misidentification may be frequent, especially if morphological taxonomic keys are solely used to differentiate species (Padial et al., 2010).

Even though morphological characters are widely used for species identification, some of the phenotypic traits that are easiest to be recognized visually often fail to delimit species accurately (Arribas et al., 2012; Lecocq et al., 2015). For example, several studies on insects, including flies (Grella et al., 2015), butterflies (Garzón-Orduña et al., 2018), and bees (Carolan et al., 2012; Ferrari & Melo, 2014; Huang et al., 2015) have shown that traits like color can be hard to interpret as diagnostic characters for species identification. Color traits may exhibit high rates of polymorphism due to environmental influences or genetic differences at a single single or multiple loci (Miyanaga et al., 1999; Uy et al., 2009; White & Kemp, 2016; Hines et al., 2017). Still, color is commonly used as a trait in taxonomic keys for many organisms, including bees, birds, and dragonflies among many others (Michener, 2007; McKay et al., 2014; Rodrílguez et al., 2015).

The advent of molecular biology has led to the development of diagnostic techniques that complement morphological species identification and may ameliorate some of the challenges with morphological taxonomic delimitation. DNA barcoding is a common molecular technique for species determination and involves the amplification of a highly variable region of the genome through PCR (polymerase chain reaction) (Hajibabaei et al., 2007). In animals, the mitochondrial gene cytochrome c oxidase subunit I (COI) is the marker of choice for DNA barcoding because it can be easily amplified with universal primers across a large number of taxa (Hebert et al., 2003), and is likely involved in the process of speciation (Hill, 2016). COI fragments are aligned and genetic distances between individuals are calculated and used as diagnostic boundaries for species. Low genetic distance among individuals indicates low divergence suggesting the presence of genetic diversity within one evolutionary lineage. On the other hand, large genetic distances among individuals may indicate the presence of independent evolutionary lineages; usually identified through a barcoding gap (Hebert et al., 2004; Čandek & Kuntner, 2015).

Despite its known intraspecific variability, color has been a widely used character among bee taxonomists for species descriptions and dichotomous keys (Michener, 2007; Colla et al., 2011; Ascher & Pickering, 2018). Some authors argue that because coloration has a well-characterized genetic basis, integument or hair color can be safely used as a morphological character for species identification (Nemésio, 2009). However, color polymorphism is well characterized in several bee groups including bumble bees (Hines & Williams, 2012; Lecocq et al., 2015; Duennes et al., 2017), orchid bees (Ferrari & Melo, 2014) and sweat bees (Miyanaga et al., 1999).

A challenging taxonomic identification involving color characters occurs within the eucerine bees of *Melissodes* genus. A taxonomic description of Urban (1973) organized into a dichotomous key the important morphological characters for species differentiation of South American *Melissodes* bees, besides had discussed the many taxonomic classifications proposed by previous authors. According to the author, there are two sympatric *Melissodes* species in Brazil, *Melissodes nigroaenea* (Smith) and *Melissodes sexcincta* (Lepeletier), whose females can be differentiated mainly by the fullness of the bristle band on the second tergum (TII), but also by the color of mesosoma hair, and the presence of maxillary subapical yellow spots.

In a recent survey of bee pollinators in cotton (*Gossypium hirsutum*) farmlands from three regions in Brazil, we collected a large number of female bees that were identified as *M. nigroaenea* based in the dichotomous key of Urban (1973). Our specimens showed two distinct color-morphs of mesosoma (yellow and black) in all populations, and few individuals showed variation in the bristle band in TII that was not expected for *M. nigroaenea*. However, the proper identification of the individuals with these unexpected patterns of bristle bands was not supported by the use of the mesosoma color character, what brought us some concern about dealing or not with more than one evolutionary unit in our bee sampling.

Using the DNA barcoding approach and the phylogenetic species concept, in which we consider a species as the smallest aggregation of sexual population or assexual lineages that presents a unique combination of character states (Wheeler & Platnick, 2000), we tested alternative hypotheses about the interpretation of color variation in *M. nigroaenea* for species identification. Specifically, we investigate whether different color morphs we sampled correspond to one or more evolutionary units by quantifying the genetic distance of these individuals using the COI barcoding region. Our hypothesis is that, if significant differences in COI sequences underpin variation in *M. nigroaenea* coloration, individuals identified as *M. nigroaenea* actually comprise two evolutionarily distinct lineages of bees. If instead we observe nominal genetic variation between individuals with distinct color-morphotypes, our alternative hypothesis is to attribute this variation to color polymorphism.

Material and Methods

Species description

M. nigroaenea (Fig 1) is a eucerine bee (Apidae: Eucerini) distributed along the east coast of Brazil, Uruguay and Argentina, and interiorly in Paraguay and Brazil (Fig 2). In Brazil, *M. nigroaenea* is the most abundant native pollinator of cotton (Cardoso et al., 2007), a fiber crop which generated $ 74.1 billion USD to the Brazilian economy (Abrapa, 2017). This native bee is a highly efficient pollinator of cotton plants, transferring a larger number of pollen grains between flowers and generating more seeds per boll when compared with honey bees (*Apis mellifera* L.) (Cardoso et al., 2007).

According to Urban (1973), females of *M. nigroaenea* might be identified for having a predominant black mesosoma with some variations of yellow, presenting the second bristle brands in the TII incomplete, and the presence of maxillary subapical yellow spots. Urban (1973) also describes *M. sexcincta* as a sympatric species, whose females have a predominant yellow mesosoma, showing two complete bristle bands in the TII. We used these characters to identify 32 female specimens as *M. nigroaenea*, although we observed few individuals with variation in the bristle bands of TII (almost absent, almost complete and complete) that did not correspond with the description of this species (incomplete)

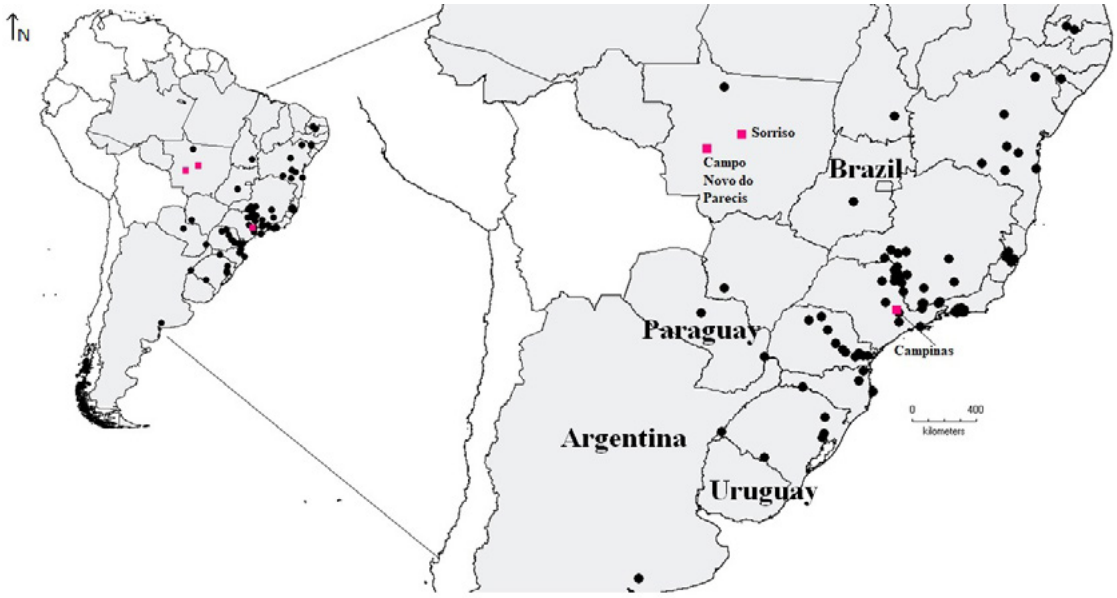

Fig 1. Map of the distribution of *Melissodes nigroaenea* (black dots), and sampling sites of this study (pink squares). Data source: Urban (1973), Discover Life database (Ascher & Pickering, 2018).

Collection sites

We collected bees in cotton fields between February and May 2016 in three different localities in Brazil (Fig 2, pink squares). Collections took place at one site within the Campinas municipality (São Paulo), one site in the Sorriso municipality (Mato Grosso), and five sites at Campo Novo do Parecis municipality (Mato Grosso) (Table 1). In 50 x 50 meter plots within cotton fields, we sampled for bees using two methods: a) netting bees visiting cotton flowers while walking along four transects in each plot, and b) setting up four blue vane traps filled with propylene glycol. We stored specimens in tubes full of 70% ethanol at 10 °C after collection.

Fig 2. Female specimens of *Melissodes nigroaenea* collected in Mato Grosso, Brazil, representing both mesosoma color morphs (a-b), and showing a continuum in the completeness of TII medial bristle bands (c-f).

Taxonomic identification

Bees were identified by Danielle C. de Luna between May and September 2016 in the Department of Biology at Faculty of Philosophy, Sciences and Literature of Ribeirão Preto at University of São Paulo (FFCLRP/USP). Identifications were based on the taxonomic key to the genus *Melissodes* developed by Urban (1973), and the reference specimens from the Entomological Collection "Prof. J. M. F. Camargo" (RPSP), University of São Paulo.

DNA extraction and quantification

We followed a protocol developed by (Boyce et al., 1989) in order to extract genomic DNA from specimens. In a 1.5 mL tube containing 4 legs of a specimen and 4 metallic beads, we added a solution containing 700 mL CTAB 2% buffer [2% (w/v) CTAB; 1.4 M NaCl, 20 mM EDTA pH 8.00; 100 mM Tris-HCl pH 8.00; 1% (w/v) 40,000 PVP; pH 8.3], 2 µL of beta-mercaptoethanol and 10 µL of proteinase K at 65 °C. Samples were then macerated and incubated at 65 °C for

Table 1. Collection sites, coordinates, color of mesosoma and GenBank accession number of the specimens used in this study. * Individuals with variation in the second bristle band of TII not expected for *M. nigroaenea* (almost absent, few or complete)

Collection Site	Coordinate	Specimen	Color of Mesosoma	GenBank Accession
Campo Novo do Parecis				
Fazenda Chapada	-13.7873667; -57.5666667	CNCHA_1590	Yellow	MH667931
Fazenda Chapada	-13.7873667; -57.5666667	CNCHA_BV575	Black	MH667928
Fazenda Gaúcha	-13.7526781; -57.6417054	CNGAU_1351	Black	MH667923
Fazenda Gaúcha	-13.7526781; -57.6417054	CNGAU_1362	Black	MH667934
Fazenda Gaúcha	-13.7526781; -57.6417054	CNGAU_1363	Black	MH667924
Fazenda Gaúcha	-13.7526781; -57.6417054	CNGAU_1380	Black	MH667925
Fazenda Gaúcha	-13.7526781; -57.6417054	CNGAU_1387	Yellow	MH667926
Fazenda Gaúcha	-13.7526781; -57.6417054	CNGAU_1389	Black	MH667927
Fazenda Graciosa	-13.4755948; -57.9402887	CNGRA_1011	Black	MH667906
Fazenda Graciosa	-13.4755948; -57.9402887	CNGRA_1013	Black	MH667909
Fazenda Graciosa	-13.4755948; -57.9402887	CNGRA_1018	Black	MH667903
Fazenda Graciosa	-13.4755948; -57.9402887	CNGRA_1049	Black	MH667912
Fazenda Graciosa	-13.4755948; -57.9402887	CNGRA_1061	Yellow	MH667904
Fazenda Graciosa	-13.4755948; -57.9402887	CNGRA_1066	Yellow	MH667907
Fazenda Graciosa	-13.4755948; -57.9402887	CNGRA_1072	Yellow	MH667910
Fazenda Ramada	-13.6845448; -57.8804554	CNRAM_1106	Yellow	MH667913
Fazenda Ramada	-13.6845448; -57.8804554	CNRAM_1111	Yellow	MH667914
Fazenda Ramada	-13.6845448; -57.8804554	CNRAM_1112	Black	MH667915
Fazenda Ramada	-13.6845448; -57.8804554	CNRAM_1122*	Black	MH667916
Fazenda Ramada	-13.6845448; -57.8804554	CNRAM_1140	Yellow	MH667917
Fazenda Ramada	-13.6845448; -57.8804554	CNRAM_1152	Black	MH667918
Fazenda Ramada	-13.6845448; -57.8804554	CNRAM_1169	Black	MH667919
Fazenda Ramada	-13.6845448; -57.8804554	CNRAM_1184*	Black	MH667920
Fazenda Ramada	-13.6845448; -57.8804554	CNRAM_1191*	Yellow	MH667921
Fazenda Ramada	-13.6845448; -57.8804554	CNRAM_1211	Yellow	MH667922
Fazenda Santa Teresinha	-13.5521948; -57.8435054	CNSTE_925	Black	MH667905
Fazenda Santa Teresinha	-13.5521948; -57.8435054	CNSTE_994	Black	MH667908
Fazenda Santa Teresinha	-13.5521948; -57.8435054	CNSTE_997	Yellow	MH667911
Sorriso				
Fazenda Primavera	-12.8651948; -55.8820887	SOPP1_BV182*	Yellow	MH667929
Fazenda Primavera	-12.8651948; -55.8820887	SOPP1_BV183	Black	MH667932
Campinas				
Fazenda Santa Elisa	-22.8662783; -47.0770554	CPSEL_1626	Yellow	MH667933
Fazenda Santa Elisa	-22.8662783; -47.0770554	CPSEL_BV588	Black	MH667930

60 min. We then added 600 µL chloroform: isoamyl alcohol (24:1) to the tubes and centrifuged at 14,000 rpm for 10 min to separate the aqueous and organic phases, and the upper aqueous phase transferred to a new 1.5 ml tube. After adding 400 µL of cold isopropanol, the samples were incubated at -20 °C for 60 min to precipitate the DNA, followed by a centrifugation at 14,000 rpm for 10 min to collect the DNA pellet, which was subsequently washed with 70% and 95% EtOH. The pellet was then air dried for approximately 2 hours. Finally, 44 µL ultrapure water and 1 µL of pure RNAse (20 mg/ µL) were added, and the samples incubated for 3 hours at 37 °C. To approximate the quantify of DNA in the samples, aloquots were run on a 1% (w/v) agarose gel and band intensities compared to known amounts in bands of a 100 bp DNA ladder.

DNA barcoding

We performed test PCR amplifications with two combinations of COI primers, Jerry-Pat (Simon et al., 1994) and HCO-LCO (Folmer et al., 1994). We made a 25 µL reaction mix containing around 20 ng of genomic DNA, 2 µL of forward primer (5 pmol/µL), 2 µL of reverse primer (5 pmol/µL), 0.5 µL of dNTP mix (10 mM), 2.5 µL of 10× Standard Taq Reaction Buffer, 2 µL of $MgCl_2$ (25 mM), and 1 U of Taq DNA polymerase (Fermentas, Thermo Fisher Scientific, Pittsburgh, Pennsylvania, USA). PCR programs for both primer pairs consisted of an initial denaturation step of three minutes at 94 ºC, followed by 40 cycles of 30 seconds at 94 ºC, 30 seconds for annealing at the specific temperature for each primer (tested with 48, 50, 51 and 52 ºC) and 1 min at 72 ºC, followed by a final extension step of 10 min at 72 ºC. We used a 1% (w/v) agarose gel to corroborate the amplification products. We used a Qiagen PCR purification kit to clean the PCR products and used bidirectional Sanger sequencing at the University of Illinois at Urbana-Champaign Core Sequencing Facility (USA).

Alignment of sequencing data

Raw sequences were edited and aligned in the software Geneious (Kearse et al., 2012). In addition to the 32 COI sequences from our study, we downloaded six COI sequences of other *Melissodes* species from GenBank and added them to the alignment (Table 2). To select the optimal model of nucleotide evolution for phylogenetic reconstruction, we used the python program PartitionFinder2 (Lanfear et al., 2017). We ran MrBayes (Ronquist & Huelsenbeck, 2003) using the best nucleotide model to estimate the phylogenetic relationships among individuals using a Bayesian inference. We adopted a sample size of 10^7 generations and a burn-in of 25% of the trees, sampling every 1,000 generations with Markov Chain Monte Carlo (MCMC) methods, which generated a consensus tree with high branch support. Our alignment file was also read into MEGA7 (Kumar et al., 2016) to estimate the pairwise genetic p-distance between our samples using 10,000 bootstraps.

Table 2. Taxa, reference paper and GenBank accession number of COI sequences of additional *Melissodes* species found in GenBank.

Taxa	Reference	GenBank accession number
Melissodes agilis	Dorchin et al. (2018)	MG251042.1
Melissodes desponsa	Dorchin et al. (2018)	MG251044.1
Melissodes druriella	Hebert et al. (2016)	KR801296.1
Melissodes ecuadorius	Packer and Ruz (2017)	KX820894.1
Melissodes illata	Sheffield et al. (2009)	FJ582332
Melissodes paroselae	Dorchin et al. (2018)	MG251041.1

Results

We identified 13 yellow and 19 black individuals among the 32 samples we collected. Even though Urban (1973) described the mesosoma color variation as a continuum, we found two discrete coloration (yellow and black, Fig 2a-b) among the individuals we collected in all populations from the 3 geographic regions. We also found variation in four individuals in the completeness of the second bristle band in the TII as described by Urban (1973). Two individuals presented almost no bristal bands (CNRAM_1122, Fig 2c, and CNRAM_1184), one presented few bristle bands in the lateral (SOPP1_BV182. Fig 2d), and one individual presented complete bristle bands (CNRAM_1191, Fig 2f). Most individuals presented incomplete bristle bands, like CNGAU_1387 (Fig 2e).

PCR reactions with the primer pair LCO 1490/HCO 2198 were optimized for our specimens at an annealing temperature of 50° C, producing a single distinct band at 710 bp. The primer pair Jerry-Pat failed to amplify from our specimens. We identified GTR+G as the best nucleotide substitution model for our data. The consensus tree obtained from MrBayes shows that the COI fragments group both color morphs into one highly supported clade (Posterior Probability = 1) sister to *Melissodes ecuadorius* (Bertoni and Schrottky), a species distributed in Ecuador, Peru, and Chile.

We found that the average pairwise genetic p-distance within yellow individuals was 0.56 % (± 0.20 %), between black individuals was 0.86 % (± 0.26 %) while between yellow and black individuals was 0.73% (± 0.23%) (Table 3). This level of genetic differentiation in mitochondrial DNA does not support the presence of two evolutionarily independent units between the different mesosoma colors. The average pairwise genetic distances among *M. nigroaenea* and other *s*pecies were between 8% and 11.5% (Table 3).

Discussion

Both pairwise genetic distances and the Bayesian phylogenetic reconstruction supported that *M. nigroaenea* individuals with yellow and black coloration in the mesosoma belong to the same evolutionary unit (Fig 3a-b; Table 3). Pairwise genetic distances within and between the two color-morphs were below 3%, considered a threshold for

differentiating species in other insect orders (Hebert et al., 2003). These results indicate that the yellow and black color morphs are a polymorphism present in sympatric populations of *M. nigroaenea.* Additionally, we also observed significant variation in the bristle band fullness of TII in four individuals, but the variation in this character was more continue than the observed variation in the color of the mesosoma (Fig 3c-f).

Taxonomic delimitations in the tribe Eucerini can be challenging primarily due to the morphological intergradation — meaning presence of intermediate characters (Terrell, 1963) — among taxa (Michener, 2007; Dorchin et al., 2018). In the *Melissodes* species description proposed by Urban (1973), the color of the mesosoma is an important character to differentiate female *M. nigroaenea* (black) from *M. sexcincta* (yellow). However, this same author recognizes the presence of variation in the mesosoma coloration in *M. nigroaenea*, suggesting possible intergradation in this diagnostic character. Another taxonomic character mentioned by Urban (1973) to differentiate females of both species was the bristle bands in the TII, in which we observed a continuous variation not expected for *M. nigroaenea*, from the almost absence to the fullness of the second bristle band in TII. We identified four variants for this character, of which three are from Fazenda Ramada (Campo Novo do Parecis region), and one from Fazenda Primavera (Sorriso region), that do not constitute a separated evolutionary unit based in our analysis. Although we might suggest the morphological intergradation for this character, further studies with more individuals from different regions will support if the bristle bands in TII could also be considered a polymorphic character for *M. nigroaenea*.

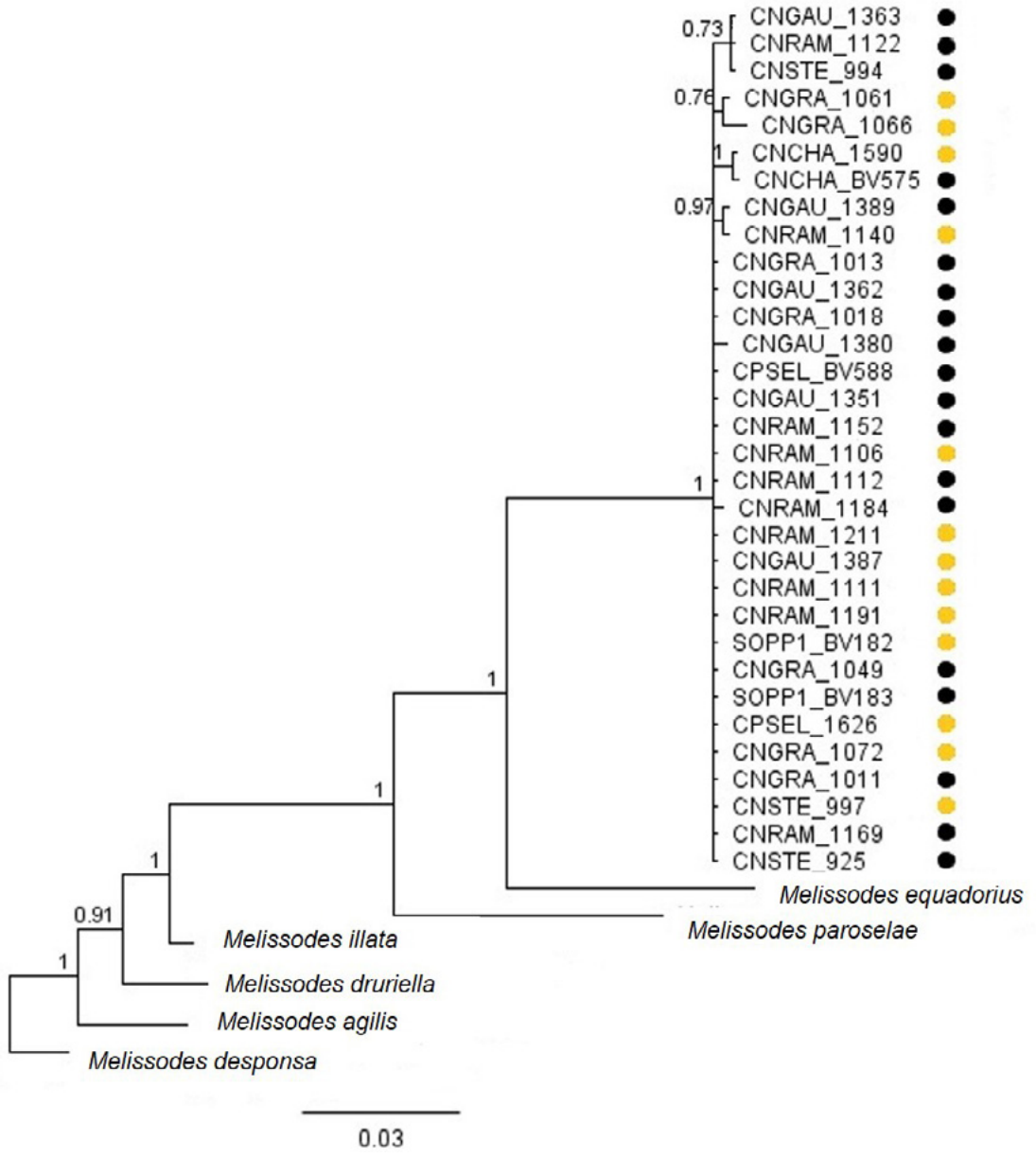

Fig 3. Consensus phylogenetic tree based on Bayesian inference generated from COI sequences for the 32 yellow and black morphs studied, plus six additional COI sequences for other *Melissodes* genus found in GenBank. The numbers over the branches correspond to posterior probability values. The scale in the bottom (0.03) means the number of substitutions per site.

Table 3. Average pairwise genetic p-distance between each color morph and other *Melissodes* species, with their respective average standard error estimates. (*) Average pairwise genetic p-distance within each color morph, with their respective average standard error estimates.

	Melissodes nigroaenea black	*Melissodes nigroaenea* yellow	*Melissodes ecuadorius*	*Melissodes illata*	*Melissodes desponsa*	*Melissodes druriella*	*Melissodes paroselae*	*Melissodes agilis*
Melissodes nigroaenea black	0.86 % (± 0.26 %)*	0.73 % (± 0.23 %)	8.03 % (± 1.18 %)	9.74 % (± 1.31 %)	11.00 % (± 1.34 %)	11.00 % (± 1.39 %)	11.30 % (± 1.43 %)	11.50 % (± 1.42 %)
Melissodes nigroaenea yellow	0.73 % (± 0.23 %)	0.56 % (± 0.20 %)*	8.01 % (± 1.19 %)	9.59 % (± 1.31 %)	10.87 % (± 1.35 %)	10.90 % (± 1.39 %)	11.29 % (± 1.44 %)	11.50 % (± 1.43 %)

Urban (1973) also made a description about the dentiform male genitalia for *Melissodes* species identification. According to the author, males of *M. nigroaenea* presents the genitalia with a cut near the valve, while *M. sexcincta* does not present this cut. However, despite of the reliance on male genital characters to confirm the taxonomy in South American *Melissodes* bees, our samples were composed mostly by females, while the few males we found were not presented in all populations. Thus, this morphological character was not considered for analyses, but may merit further study, especially in combination with DNA barcoding.

Additional studies using molecular techniques to resolve taxonomic issues within bees have also concluded that color should not be used as a primary character for taxonomic classification. Ferrari and Melo (2014) found that the integument color of three species of *Euglossa* orchid bees present geographic variation in Brazil, with both blue and green colors observed. However, the authors did not find phylogenetic support for the independence of the three *Euglossa* species, nor did they observe a vast enough genetic distance to justify separating both color morphs into different species. Another group of bees known for high variability in coloration pattern are the bumble bees. Huang et al. (2015) found nine distinct color patterns for female bumble bees in different regions of China, but all color morphs clustered together as a monophyletic group in the Bayesian analysis, belonging to *Bombus koreanus* (Skorikov). Carolan et al. (2012) tested the hypothesis that color patterns could reliably distinguish three *Bombus* in the *lucorum* complex from one another when compared to COI sequence data, and found that morphological data often resulted in incorrect classification of individual specimens, due to the cryptic nature of the *lucorum* complex. However, taxonomic characters can be informative to identify species, especially when they are interpreted in conjunction with another type of dataset like DNA barcoding (Freitas et al., 2018).

Here, we confirmed a mesosoma color polymorphism in *M. nigroaenea,* an important pollinator of cotton plantations in Brazil. Our results suggest that this species exhibits yellow and black individuals in multiple areas across its distribution. Sampling a higher number of individuals from more populations and male individuals in future studies will contribute to a better understanding of the ubiquity of this polymorphism in *M. nigroaenea*, and also to confirm if the bristle bands in the TII might be considered a polymorphic character. In addition, more molecular markers will be necessary to determine whether these two color-morphs are the result of recent incipient speciation. Species delimitation studies using museum materials identified as *M. sexcincta* may assist in clarifying whether this species and *M. nigroaenea* and are two evolutionary independent entities or should be synonymized.

Acknowledgements

We would like to thank to São Paulo Research Foundation - FAPESP for funding this research (Grant number 2014/50738-9), and the Coordination for the Improvement of Higher Education Personnel - CAPES (Programa Biologia Computacional, Brazil grant number 1572813, and US Grant number 060/2017-08) for the Post-doctoral scholarship of C. Grando at University of Campinas and Penn State University, respectively. We thank to Danielle C. de Luna for the precious help with bee collections and the identifications, as well as Prof. Eduardo A. B. de Almeida for the use of his lab and the access to the Entomological Collection "Prof. J. M. F. Camargo" (RPSP), University of São Paulo, during the identification. We are also grateful to Instituto Matogrossense do Algodão (IMAmt) for making possible our bee collections in cotton farms of the State of Mato Grosso (Brazil). Last, but extremely important, we would like to express our admiration and gratitude for all the contributions of Prof. Danuncia Urban for the taxonomy of bees in Brazil.

Authors' Contributions

CG, MMLU and MIZ conceived and designed the study; CG conducted the field sampling; CG, SC, NG and WW generated the molecular data; PA photographed bee individuals; CG and NDA conducted the data analyses; CG, NDA, MIZ, and MMLU drafted the manuscript. All authors gave final approval of the manuscript for publication.

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