



## 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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### 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 (Vodá 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 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; Rodríguez 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* (Lepelletier), 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 asexual 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 bands 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 |
|------------------------------|--------------------------|--------------|-------------------|-------------------|
| <b>Campo Novo do Parecis</b> |                          |              |                   |                   |
| 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          |
| <b>Sorriso</b>               |                          |              |                   |                   |
| Fazenda Primavera            | -12.8651948; -55.8820887 | SOPP1_BV182* | Yellow            | MH667929          |
| Fazenda Primavera            | -12.8651948; -55.8820887 | SOPP1_BV183  | Black             | MH667932          |
| <b>Campinas</b>              |                          |              |                   |                   |
| 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  $\mu\text{L}$  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  $\mu\text{L}$  of cold isopropanol, the samples were incubated at  $-20\text{ }^{\circ}\text{C}$  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  $\mu\text{L}$  ultrapure water and 1  $\mu\text{L}$  of pure RNase (20 mg/  $\mu\text{L}$ ) were added, and the samples incubated for 3 hours at  $37\text{ }^{\circ}\text{C}$ . To approximate the quantify of DNA in the samples, aliquots 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  $\mu\text{L}$  reaction mix containing around 20 ng of genomic DNA, 2  $\mu\text{L}$  of forward primer (5 pmol/ $\mu\text{L}$ ), 2  $\mu\text{L}$  of reverse primer (5 pmol/ $\mu\text{L}$ ), 0.5  $\mu\text{L}$  of dNTP mix (10 mM), 2.5  $\mu\text{L}$  of 10 $\times$  Standard Taq Reaction Buffer, 2  $\mu\text{L}$  of  $\text{MgCl}_2$  (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  $94\text{ }^{\circ}\text{C}$ , followed by 40 cycles of 30 seconds at  $94\text{ }^{\circ}\text{C}$ , 30 seconds for annealing at the specific temperature for each primer (tested with 48, 50, 51 and  $52\text{ }^{\circ}\text{C}$ ) and 1 min at  $72\text{ }^{\circ}\text{C}$ , followed by a final extension step of 10 min at  $72\text{ }^{\circ}\text{C}$ . 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 |
|------------------------------|-------------------------|--------------------------|
| <i>Melissodes agilis</i>     | Dorchin et al. (2018)   | MG251042.1               |
| <i>Melissodes desponsa</i>   | Dorchin et al. (2018)   | MG251044.1               |
| <i>Melissodes druriella</i>  | Hebert et al. (2016)    | KR801296.1               |
| <i>Melissodes ecuadorius</i> | Packer and Ruz (2017)   | KX820894.1               |
| <i>Melissodes illata</i>     | Sheffield et al. (2009) | FJ582332                 |
| <i>Melissodes paroselae</i>  | 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  $50\text{ }^{\circ}\text{C}$ , 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 % ( $\pm 0.20\%$ ), between black individuals was 0.86 % ( $\pm 0.26\%$ ) while between yellow and black individuals was 0.73% ( $\pm 0.23\%$ ) (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 species 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



**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.

|  | <i>Melissodes nigroaenea</i><br>black | <i>Melissodes nigroaenea</i><br>yellow | <i>Melissodes ecuadorius</i> | <i>Melissodes illata</i> | <i>Melissodes desponsa</i> | <i>Melissodes druriella</i> | <i>Melissodes paroselae</i> | <i>Melissodes agilis</i> |
|--|---------------------------------------|--|------------------------------|--------------------------|----------------------------|-----------------------------|-----------------------------|--------------------------|
| <i>Melissodes nigroaenea</i><br>black  | 0.86 %<br>(± 0.26 %)*                 | 0.73 %<br>(± 0.23 %)                   | 8.03 %<br>(± 1.18 %)         | 9.74 %<br>(± 1.31 %)     | 11.00 %<br>(± 1.34 %)      | 11.00 %<br>(± 1.39 %)       | 11.30 %<br>(± 1.43 %)       | 11.50 %<br>(± 1.42 %)    |
| <i>Melissodes nigroaenea</i><br>yellow | 0.73 %<br>(± 0.23 %)                  | 0.56 %<br>(± 0.20 %)*                  | 8.01 %<br>(± 1.19 %)         | 9.59 %<br>(± 1.31 %)     | 10.87 %<br>(± 1.35 %)      | 10.90 %<br>(± 1.39 %)       | 11.29 %<br>(± 1.44 %)       | 11.50 %<br>(± 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* are two evolutionary independent entities or should be synonymized.

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### 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.

### References

Arribas, P., Abellán, P., Velasco, J., Bilton, D.T., Millán, A. & Sánchez-Fernández, D. (2012). Evaluating drivers of vulnerability to climate change: a guide for insect conservation strategies. *Global Change Biology*, 18: 2135–2146. doi: 10.1111/j.1365-2486.2012.02691.x

- Ascher, J.S. & Pickering, J. (2017). Discover Life bee species guide and world checklist (Hymenoptera: Apoidea: Anthophila). [http://www.discoverlife.org/mp/20q?search= Apoidea](http://www.discoverlife.org/mp/20q?search=Apoidea) (accessed date: 18 April, 2018)
- Abrapa (2017). A cadeia do algodão brasileiro: safra 2016/2017. Associação Brasileira dos Produtores de Algodão, 248 p
- Austen, G.E., Bindemann, M., Griffiths, R.A. & Roberts, D.L. (2016). Species identification by experts and non-experts: comparing images from field guides. *Scientific Reports*, 6: 33634. doi: 10.1038/srep33634
- Bickford, D., Lohman, D.J., Sodhi, N.S., Ng, P.K.L., Meier, R., Winker, K., Ingram, K.K. & Das, I. (2007). Cryptic species as a window on diversity and conservation. *Trends in Ecology & Evolution*, 22: 148–155. doi: 10.1016/j.tree.2006.11.004
- Boyce, T.M., Zwick, M.E. & Aquadro, C.F. (1989). Mitochondrial DNA in the bark weevils: size, structure and heteroplasmy. *Genetics*, 123: 825–836.
- Čandek, K. & Kuntner, M. (2015). DNA barcoding gap: reliable species identification over morphological and geographical scales. *Molecular Ecology Resources*, 15: 268–277. doi: 10.1111/1755-0998.12304
- Cardoso, C.F., Silveira, F.A., Oliveira, G.M., Cavéchia, L.A., Almeida, J.P.S., Nakasu, E.Y.T., Sujii, E.R., Fontes, E.M.G., Pires, C.S.S. (2007). Principais polinizadores de *Gossypium hirsutum latifolium* cv. Delta Opal (Malvaceae), em uma localidade do Distrito Federal, Brasil. *Boletim de Pesquisa e Desenvolvimento* 212. Brasília: Embrapa Recursos Genéticos e Biotecnologia.
- Carolan, J.C., Murray, T.E., Fitzpatrick, Ú., Crossley, J., Schmidt, H., Cederberg, B., McNally, L., Paxton, R.J., Williams, P.H. & Brown, M.J.F. (2012). Colour patterns do not diagnose species: quantitative evaluation of a DNA barcoded cryptic bumblebee complex. *Plos One*, 7(1): e29251. doi: 10.1371/journal.pone.0029251
- Colla S, Richardson L. & Williams P. (2011). Bumble bees of the eastern United States. <https://www.fs.fed.us/wildflowers/pollinators/documents/BumbleBeeGuideEast2011.pdf>. (accessed date: 24 April, 2018)
- Dorchin, A., López-Urbe, M.M., Praz, C.J., Griswold, T. & Danforth, B.N. (2018). Phylogeny, new generic-level classification, and historical biogeography of the *Eucera* complex (Hymenoptera: Apidae). *Molecular Phylogenetics and Evolution*, 119: 81–92. doi: 10.1016/j.ympev.2017.10.007
- Duennes, M.A., Petranek, C., de Bonilla, E.P.D., Mérida-Rivas, J., Martínez-López, O., Sagot, P., Vandame, R. & Cameron, S.A. (2017). Population genetics and geometric morphometrics of the *Bombus ephippiatus* species complex with implications for its use as a commercial pollinator. *Conservation Genetics*, 18: 553–572. doi: 10.1007/s10592-016-0903-9
- Ferrari, B.R. & Melo, G.A.R. (2014). Deceiving colors: recognition of color morphs as separate species in orchid bees is not supported by molecular evidence. *Apidologie*, 45: 641–652. doi: 10.1007/s13592-014-0280-7
- Folmer, Black, Hoeh, Lutz & Vrijenhoek (1994). DNA primers for amplification of mitochondrial cytochrome c. *Molecular Marine Biology and Biotechnology*, 3: 294–299
- Freitas, F.V., Santos Júnior, J.E., Santos, F.R. & Silveira, F.A. (2018). Species delimitation and sex associations in the bee genus *Thygater*, with the aid of molecular data, and the description of a new species. *Apidologie*, 1–13. doi: 10.1007/s13592-018-0576-0
- Garzón-Orduña, I.J., Brower, A.V.Z., Kamilari, M., Iribar, A. & Muriene, J. (2018). Cracking the Code: Examination of species delimitations among *Hamadryas* butterflies with DNA barcodes suggests Caribbean cracker is *Hamadryas februa* Hübner (Nymphalidae: Biblidinae). *Journal of the Lepidopterists' Society*, 72: 53–73. doi: 10.18473/lepi.72i1.a6
- Grella, M.D., Savino, A.G., Paulo, D.F., Mendes, F.M., Azeredo-Espin, A.M.L., Queiroz, M.M.C., Thyssen, P.J. & Linhares, A.X. (2015). Phenotypic polymorphism of *Chrysomya albiceps* (Wiedemann) (Diptera: Calliphoridae) may lead to species misidentification. *Acta Tropica*, 141: 60–72. doi: 10.1016/j.actatropica.2014.09.011
- Hajibabaei, M., Singer, G.A.C., Hebert, P.D.N. & Hickey, D.A. (2007). DNA barcoding: how it complements taxonomy, molecular phylogenetics and population genetics. *Trends in Genetics*, 23: 167–172. doi: 10.1016/j.tig.2007.02.001
- Hebert, P.D.N., Cywinska, A., Ball, S.L. & deWaard, J.R. (2003). Biological identifications through DNA barcodes. *Proceedings of the Royal Society Biological Sciences*, 270: 313–321. doi: 10.1098/rspb.2002.2218
- Hebert, P.D.N., Penton, E.H., Burns, J.M., Janzen, D.H. & Hallwachs, W. (2004). Ten species in one: DNA barcoding reveals cryptic species in the neotropical skipper butterfly *Astraptes fulgerator*. *Proceedings of the National Academy of Sciences of the United States of America*, 101: 14812–14817. doi: 10.1073/pnas.0406166101
- Hebert, P.D.N., Ratnasingham, S., Zakharov, E.V., Telfer, A.C., Levesque-Beaudin, V., Milton, M.A., Pedersen, S., Jannetta, P. & deWaard, J.R. (2016). Counting animal species with DNA barcodes: Canadian insects. *Philosophical Transactions of the Royal Society of London. Series B, Biological Sciences*, 371(1702). doi: 10.1098/rstb.2015.0333
- Hill, G.E. (2016). Mitonuclear coevolution as the genesis of speciation and the mitochondrial DNA barcode gap. *Ecology and Evolution*, 6: 5831–5842. doi: 10.1002/ece3.2338
- Hines, H.M. & Williams, P.H. (2012). Mimetic colour pattern evolution in the highly polymorphic *Bombus trifasciatus* (Hymenoptera: Apidae) species complex and its comimics.

- Zoological Journal of the Linnean Society, 166: 805–826. doi: 10.1111/j.1096-3642.2012.00861.x
- Hines, H.M., Witkowski, P., Wilson, J.S. & Wakamatsu, K. (2017). Melanic variation underlies aposematic color variation in two hymenopteran mimicry systems. *Plos One*, 12: e0182135. doi: 10.1371/journal.pone.0182135
- Huang, J., Wu, J., An, J. & Williams, P.H. (2015). Newly discovered colour-pattern polymorphism of *Bombus koreanus* females (Hymenoptera: Apidae) demonstrated by DNA barcoding. *Apidologie*, 46: 250–261. doi: 10.1007/s13592-014-0319-9
- Kearse, M., Moir, R., Wilson, A., Stones-Havas, S., Cheung, M., Sturrock, S., Buxton, S., Cooper, A., Markowitz, S., Duran, C., Thierer, T., Ashton, B., Meintjes, P. & Drummond, A. (2012). Geneious Basic: an integrated and extendable desktop software platform for the organization and analysis of sequence data. *Bioinformatics*, 28: 1647–1649. doi: 10.1093/bioinformatics/bts199
- Kumar, S., Stecher, G. and Tamura, K. (2016). MEGA7: Molecular evolutionary genetics analysis version 7.0 for bigger datasets. *Molecular Biology and Evolution*, 33: 1870–1874. doi: 10.1093/molbev/msw054
- Lanfear, R., Frandsen, P.B., Wright, A.M., Senfeld, T. and Calcott, B. (2017). Partitionfinder 2: new methods for selecting partitioned models of evolution for molecular and morphological phylogenetic analyses. *Molecular Biology and Evolution*, 34: 772–773. doi: 10.1093/molbev/msw260
- Lecocq, T., Dellicour, S., Michez, D., Dehon, M., Dewulf, A., De Meulemeester, T., Brasero, N., Valterová, I., Rasplus, J.-Y. and Rasmont, P. 2015. Methods for species delimitation in bumblebees (Hym., Apidae, *Bombus*): towards an integrative approach. *Zoologica Scripta*, 44: 281–297. doi: 10.1111/zsc.12107
- McKay, B.D., Mays, H.L., Yao, C.-T., Wan, D., Higuchi, H. and Nishiumi, I. 2014. Incorporating color into integrative taxonomy: analysis of the varied tit (*Sittiparus varius*) complex in East Asia. *Systematic Biology*, 63: 505–517. doi: 10.1093/sysbio/syu016
- Michener, C.D. (2007). *The bees of the world*. Baltimore: Johns Hopkins University Press, 992 p
- Miyanaga, R., Maeta, Y. & Sakagami, S.F. (1999). Geographical variation of sociality and size-linked color patterns in *Lasioglossum* (*Evylaeus*) *apristum* (Vachal) in Japan (Hymenoptera, Halictidae). *Insectes Sociaux*, 46: 224–232. doi: 10.1007/s000400050138
- Nemésio, A. (2009). Orchid bees (Hymenoptera: Apidae) of the Brazilian Atlantic Forest. *Zootaxa*, 2041: 1–242.
- Packer, L., Monckton, S.K., Onuferko, T.M. & Ferrari, R.R. (2018). Validating taxonomic identifications in entomological research. *Insect Conservation and Diversity*, 11: 1–12. doi: 10.1111/icad.12284
- Packer, L. & Ruz, L. (2017). DNA barcoding the bees (Hymenoptera: Apoidea) of Chile: species discovery in a reasonably well know bee fauna with the description of a new species of *Lonchopria* (Colletidae). *Genome*, 60: 414–430. doi: 10.1139/gen-2016-0071
- Padial, J.M., Miralles, A., De la Riva, I. & Vences, M. (2010). The integrative future of taxonomy. *Frontiers in Zoology*, 7:16. doi: 10.1186/1742-9994-7-16
- Rodríguez, F.P., Sarmiento, C.E. & González-Soriano, E. (2015). Morphological variability and evaluation of taxonomic characters in the genus *Erythemis* Hagen, 1861 (Odonata: Libellulidae: Sympetrinae). *Insecta Mundi*, 0428: 1–68. Center for Systematic Entomology, Inc.
- Ronquist, F. & Huelsenbeck, J.P. (2003). MrBayes 3: Bayesian phylogenetic inference under mixed models. *Bioinformatics*, 19: 1572–1574. doi: 10.1093/bioinformatics/btg180
- Sheffield, C.S., Hebert, P.D.N., Kevan, P.G. & Packer, L. (2009). DNA barcoding a regional bee (Hymenoptera: Apoidea) fauna and its potential for ecological studies. *Molecular Ecology Resources*, 9 Suppl s1:196–207. doi: 10.1111/j.1755-0998.2009.02645.x.
- Sigovini, M., Keppel, E. & Tagliapietra, D. (2016). Open Nomenclature in the biodiversity era. *Methods in Ecology and Evolution*, 7: 1217–1225. doi: 10.1111/2041-210X.12594
- Simon, C., Frati, F., Beckenbach, A., Crespi, B., Liu, H. & Flook, P. (1994). Evolution, weighting, and phylogenetic utility of mitochondrial gene sequences and a compilation of conserved polymerase chain reaction primers. *Annals of the Entomological Society of America*, 87: 651–701.
- Terrell, E.E. (1963). Symbols and terms for morphological intergradation and hybridization. *Taxon*, 12: 105–108.
- Urban, D. (1973). As espécies sulamericanas do gênero *Melissodes* (Latreille, 1829) (Hymenoptera: Apoidea). *Revista Brasileira de Biologia*, 33: 201–220.
- Uy, J.A.C., Moyle, R.G., Filardi, C.E. & Cheviron, Z.A. (2009). Difference in plumage color used in species recognition between incipient species is linked to a single amino acid substitution in the melanocortin-1 receptor. *The American Naturalist*, 174: 244–254. doi: 10.1086/600084
- Vodá, R., Dapporto, L., Dincă, V. & Vila, R. (2015). Cryptic matters: overlooked species generate most butterfly beta-diversity. *Ecography*, 38: 405–409. doi: 10.1111/ecog.00762
- White, T.E. & Kemp, D.J. (2016). Colour polymorphism. *Current Biology*, 26: R517–R518. doi: 10.1016/j.cub.2016.03.017
- Wheeler, Q.D. & Platnick, N.I. (2000). The phylogenetic species concept (sensu Wheeler and Platnick). In Q.D. Wheeler & R. Meier (Eds.), *Species concepts and phylogenetic theory: A debate*. New York: Columbia University Press.