Using an integrative taxonomic approach to delimit a sibling species, *Mycetomoellerius mikromelanos* sp. nov. (Formicidae: Attini: Attina)

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Abstract

The fungus-growing ant *Mycetomoellerius* (previously *Trachymyrmex*) *zeteki* (Weber (1940) has been the focus of a wide range of studies examining symbiotic partners, garden pathogens, mating frequencies, and genomics. This is in part due to the ease of collecting colonies from creek embankments and its high abundance in the Panama Canal region. The original description was based on samples collected on Barro Colorado Island (BCI), Panama (BCI). However, most subsequent studies have sampled populations on the mainland 15 km southeast of BCI. Herein we show that two sibling ant species live in sympatry on the mainland: *Mycetomoellerius mikromelanos* sp. nov. Cardenas, Schultz, and& Adams and *M. zeteki.* This distinction was originally based on behavioral differences of workers in the field and on queen morphology (*M. mikromelanos* workers and queens are smaller and **black** while those of *M. zeteki* are larger and red). Authors frequently refer to either species as "*M.* cf. *zeteki,*" indicating uncertainty about identity. We used an integrative taxonomic approach to resolve this, examining worker behavior, chemical profiles of worker volatiles, molecular markers, and morphology of all castes. For the latter, we used conventional taxonomic indicators from nine measurements, six extrapolated indices, and morphological characters. We document a new observation of a Diapriinae (Hymenoptera: Diapriidae) parasitoid wasp parasitizing *M. zeteki*. Finally, we discuss the importance of vouchering in dependable, accessible museum collections and provide a table of previously published papers to clarify the usage of the name *T. zeteki*. We found that thcat most reports of *M. zeteki* or *M.* cf. *zeteki*—including a genome—actually refer to the new species *M. mikromelanos*.

Introduction

Fungus-growing ants (Hymenoptera: Formicidae: ~~Tribe~~ Attini: ~~Subtribe~~ Attina; Ward et al., 2015), here referred to as "attine" ants, cultivate mutualistic fungus gardens using sophisticated agricultural practices (Weber, 1958a). This clade of 240 extant described species has been tending and feeding cultivated fungi for ca. 60 million years (Branstetter et al., 2017). Because fungus-growing ants have been focal taxa of studies in evolutionary biology, including mating systems (Baer & Boomsma, 2004; Boomsma, 2007), symbiotic networks (Mueller, Rehner & Schultz, 1998; Currie, Mueller & Malloch, 1999), social parasitism (Adams et al., 2013), host fidelity (Mehdiabadi et al., 2012), and genome evolution (Nygaard et al., 2016), it is imperative that the taxonomy of attine ants accurately reflects their evolutionary history. Diverse studies indicate the existence of many undescribed species (Schultz & Meier, 1995; Schultz, Bekkevold & Boomsma, 1998; Rabeling et al., 2007; Schultz & Brady, 2008; Mehdiabadi et al., 2012; Ješovnik et al., 2013; Sosa-Calvo et al., 2018; Solomon et al., 2019) and alpha-taxonomic work has been steadily carried out by many taxonomists (Mayhé-Nunes & Brandão, 2002, 2005, 2007; Sosa-Calvo & Schultz, 2010; Ješovnik et al., 2013; Rabeling et al., 2015; Ješovnik & Schultz, 2017; Sosa-Calvo et al., 2017, 2018), in fact an average of 2.4 new attine species have been described per year from 1995 to 2019 (Table S1, e.g., Schultz et al.~~,~~ 2002; Ješovnik et al.~~,~~ 2013; Sánchez-Peña et al.~~,~~ 2017).

Taxonomists have informally split the attines into lower and higher fungus-growing ants based on varying systems of fungus-farming agriculture (Schultz & Brady, 2008). The lower attines cultivate a diversity of undomesticated fungal cultivars, while the higher attines generally cultivate a closely related lineage of domesticated (i.e., obligately mutualistic) fungal species, including *Leucoagaricus gongylophorus* (Möller) Singer 1986 (Schultz ~~and~~& Brady~~,~~ 2008; Branstetter et al.~~,~~ 2017; but see Mueller et al.~~,~~ 2018). The most derived and familiar higher-attine genera consist of the leaf-cutting ants~~,~~ *Atta* Fabricius 1804 and *Acromyrmex* Mayr 1865, which largely cut fresh plant material for their gardens. However, the other higher-attine genera consist of *Sericomyrmex* Mayr 1865, *Trachymyrmex* Forel 1893, *Xerolitor* Sosa-Calvo, Schultz, Jesovnik, Dahan~~,~~ ~~and~~& Rabeling~~,~~ 2018, *Mycetomoellerius* Solomon, Rabeling, Sosa-Calvo~~,~~ ~~and~~& Schultz 2019, and *Paratrachymyrmex* Solomon, Rabeling, Sosa-Calvo~~,~~ ~~and~~& Schultz 2019.

These ~~non-leaf-cutting,~~ other higher-attine ants~~-~~, hereafter referred to as higher attines, are phylogenetically intermediate between the lower-attine and leaf-cutting ants (Brandão & Mayhé-Nunes, 2007)~~.~~, and

~~These non-leaf-cutting, higher-attine ants, hereafter referred to as higher attines,~~ share natural history traits with both ~~the lower attines and leaf-cutting ants~~. Similar to leaf-cutting ants, some higher attines have also been observed cutting fresh plant material for their gardens (Weber, 1972; Schultz & Meier, 1995; Leal & Oliveira, 2000; Mayhé-Nunes & Brandão, 2005; Brandão & Mayhé-Nunes, 2007). Otherwise, much like lower attines, higher attines typically harvest fallen flowers, fruits, leaves, small twigs, seeds, and caterpillar frass (Lizidatti, 2006; De Fine

Licht & Boomsma, 2010; Ronque, Feitosa & Oliveira, 2019). Unlike lower-attine workers that are typically monomorphic, workers in *Mycetomoellerius*, *Paratrachymyrmex,* and *Trachymyrmex* tend to be weakly polymorphic (Weber, 1958a; Beshers & Traniello, 1996; Brandão & Mayhé-Nunes, 2007; Rabeling et al., 2007). It is this variability in worker morphology, coupled with species descriptions based on a few workers (Weber, 1940), sampling bias (see Mueller et al., 2018), and inconsistent voucher deposition that have led to incorrect or incomplete species identifications (Appendix Table 8). This is evident in the recent splitting of the paraphyletic genus *Trachymyrmex* into *Trachymyrmex*, *Paratrachymyrmex*, and *Mycetomoellerius*~~,~~ (Solomon et al., 2019).

Mycetomoellerius zeteki (Weber, 1940)~~, previously *Trachymyrmex zeteki (Solomon et al., 2019)*~~, exemplifies the need for taxonomic clarity in the attines. Abundant and easily collected in the Panama Canal Zone, *M. zeteki* has been included in a large breadth of work (Appendix Table 8). Notable research employing *M. zeteki* includes discovering the function of actinomycete bacteria in the fungus-growing ants (Currie, Mueller & Malloch, 1999), describing the evolutionary transition from single to multiple mating in the fungus-growing ants (Villesen et al., 2002), and the reciprocal evolution of ant and fungal genomes in the fungus-growing ant symbiosis (Nygaard et al., 2016). Despite this attention to its biology, even *M. zeteki* has remained taxonomically ambiguous. For example, in a phylogenetic analysis of ~~A~~actinomycetes bacteria associated with attine ants, three samples form a polytomy containing *M.* sp. 'Funnel', an undetermined *Mycetomoellerius* sp., and *M. zeteki sensu stricto* were included (Cafaro & Currie, 2005). It has been speculated that the current definition of *M. zeteki* may include a cryptic, possibly sibling species based on behavioral (Adams and Schultz unpublished), morphological (Adams et al., 2012b), molecular (Solomon et al., 2019), and chemical differences (Adams, Jones & Jeter, 2010; Adams et al., 2012a). This uncertainty surrounding *M.* cf. *zeteki* has ramifications given its significant historical contributions to fungus-growing ant research (Appendix Table 8). To resolve this, we use an integrative approach to clarify the taxonomy of *M. zeteki* by reexamining morphological characters, comparing old and new collections, examining morphometrics, adapting a comparative behavioral method for worker tempo, and chemically analyzing worker volatile compounds. Based on these diverse data, we recognize two species: *M. zeteki* and *Mycetomoellerius mikromelanos* sp. nov. We provide a diagnosis and description of *M. mikromelanos* sp. nov., describe the *M. zeteki* gyne wings and the morphological characters of *M. zeteki* males, determine the identity of the published *M. zeteki* genome, suggest corrections for the misidentification of voucher specimens in published research, and discuss the implications of our improved species-level definitions.

Materials & Methods

Sample collections

Colonies of *M. mikromelanos* sp. nov. and *M. zeteki* were collected at the start of the wet season in 2017 and 2018 in the Canal Zone of the Republic of Panama (9.12007, -79.7317). Colony collection and field work were approved by The Smithsonian Tropical Research Institute as part

of the “Behavioral Ecology and Systematics of the Fungus-growing Ants and Their Symbionts (#4056)" project and the Autoridad Nacional del Ambiente y el Mar (Permiso de Colecta Científica 2017: SPO-17-173, 2018: SE/AB-1-18). Samples were collected by excavating only the first (i.e., upper) chamber of the nest to ensure colony survival. Of those excavated in 2018, 16 of 30 colonies were collected into five-dram vials (BioQuip, Cat. No. 8905, California, United States) and transferred to Petri dishes lined with moist cotton fiber for observations while in Panama. Vouchers of ca. 10 or more workers and fungus gardens from each nest were collected in 95% EtOH. Live colonies were brought back to The Ohio State University to a United States Department of Agriculture Animal and Plant Health Inspection Service Approved Facility (OSU; Columbus Ohio, USA; APHIS permit P526P-16-02785; facility #4036), where they were transferred to permanent nest boxes (as in Sosa-Calvo et al., 2015).

Taxonomy & Morphometrics

We used a Wild M-5 microscope equipped with an ocular micrometer to examine specimens for morphological characters that unambiguously separate the two species. We also took morphological measurements of 171 workers (n = 54 *M. zeteki*, n = 117 *M. mikromelanos* sp. nov.), 53 queens (n = 28 *M. zeteki*, n = 25 *M. mikromelanos* sp. nov.), and 43 males (n = 22 *M. zeteki*, n = 21 *M. mikromelanos* sp. nov.) using standard morphometrics (Table 1). Of these samples, we included two synonymized *M. balboai* syntypes (‘cotypes’) and one additional specimen identified as *M. balboai*. Including this junior synonym of *M. zeteki* (proposed by Weber, 1958b) was necessary to confirm that *M. mikromelanos* is not *M. balboai*. Upon confirmation, these samples were included as *M. zeteki* in further analyses. Terminology for the temple and malar areas follows that of Boudinot et al. (~~Boudinot, Sumnicht & Adams,~~ 2013) and for sculpturing that of Harris (~~Harris,~~ 1979). Type and voucher specimens of material examined are deposited at United States National Museum (USNM), Museum of Zoology of the University of São Paulo (MZSP), Smithsonian Tropical Research Institute (STRI), and The Ohio State University Museum of Biological Diversity Triplehorn Insect Collection (OSUC).

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We partitioned specimens by caste and tested the assumption of normality for each morphometric character with a Shapiro-Wilks test. We used a Welch’s t-test for normally distributed and a Wilcoxon Rank Sum test for non-normally distributed variables to test the null

hypothesis of equal means and differences in range between both species. In the Wilcoxon Rank Sum ~~T~~test there were ties in the data, so exact p-values could not be calculated for all castes. Both the normality testing and difference of means was performed in the base R package 'stats' (R Core Team, 2017). To reduce the risk of Type I error, only measurements with a Bonferroni corrected P-value ($p < 0.003$) were included.

With our retained variables, we performed non-metric multidimensional scaling (NMDS) with the vegan R package, using the 'metaMDS' function (Oksanen et al., 2019). This function calculates the Bray-Curtis distances, applies a square root transformation, and scales the distance measures down to k dimensions. We set $k = 2$, and the analysis was run for 1,000 iterations. We subsequently produced a diagnostic Shepard plot with the 'stressplot' command from vegan. We considered our reduced dimensions acceptable if our transformed data reasonably fit the regression of the Shepard plot and if stress scores were < 0.20 (McCune & Grace, 2002). We generated NMDS plots with characters plotted as vectors and 95% confidence ellipses for each species.

Behavioral Assay~~-~~

We adapted the novel environment assay (Chapman et al., 2011) to examine the tempo, i.e., activity level, of workers of *M. zeteki* and *M. mikromelanos* sp. nov. We subsampled four colonies of each species with five trials per colony. Single workers were selected from the foraging chamber and placed in the center of a 9 cm Petri dish lined with 1 cm^2 grid paper. The ant was immediately covered with one quarter of a 4.5 cm weigh boat (referred to as "refuge" hereafter). Five-minute trials were recorded with a Sony DCR-PC109 camera, digitized from the cassette tape, and scored using Solomon Coder (Péter, 2017). We measured (1) time to initially emerge from the refuge, (2) number of squares the ant entered, and (3) time spent under the refuge after the initial emergence. To analyze the change in tempo over the trial, we produced a ratio of squares entered to time spent entering squares (i.e., not under the refuge): New Squares / (300 s – Time to Exit Refuge – Time Under Refuge – Time on Refuge) = Tempo.

To test whether tempo differed between species we used a generalized linear mixed model (GLMM, family: gamma, link function: log-link) with the fixed effect as species and random effect as the workers' colony origin. We used a GLMM to account for multiple workers sampled from the same colony. We used the package 'lme4' (Bates et al., 2015) in R (R Core Team, 2017). We compared our GLMM model to a linear mixed model (LMM), with distribution families gaussian and gamma with the appropriate link functions log-link, identity link, and inverse link functions. We selected the model with the lowest AIC values, a gamma distribution with a log-link function. We used GLMM model as it accounts for non-independent data. We subsequently checked the fit of our best model with a QQ-plot, density plot, and Shapiro-Wilk test, and examined the homoscedasticity of our data by plotting the residuals from our GLMM.

Phylogenetic analysis

We used sequence data published in Solomon et al.~~,~~ (2019; available on Dryad DOI: 10.5061/dryad.2p7r771) to confirm the identity of the published genome (Nygaard et al., 2016). We extracted sequences of *M. zeteki*, *M. mikromelanos* (listed as *Mycetomoellerius* n. sp. RMMA in Solomon et al., 2019; Table S2), and *M. turrifex* ~~Wheeler~~ (Wheeler~~,~~ 1903) from the dataset of Solomon et al. (2019) and aligned them in Geneious (version R9; Biomatters Limited, Auckland, New Zealand). We used BLAST with blastn and megablast (Altschul et al., 1990; Zhang et al., 2000; Morgulis et al., 2008) to identify quality gene regions in the published genome (Nygaard et al., 2016; GenBank accession: GCA_001594055.1). The gene for ***COI*** was removed from the analysis because ***COI*** data were missing for a subset of individuals in the data of Solomon et al. (~~Solomon et al.,~~ 2019). Megablast found no alignments and blastn found multiple scaffolds with high query cover (see ~~r~~Results, and Table S3). In Geneious, we mapped our samples to the identified reference genome scaffolds and trimmed the areas of the scaffold that did not align. Once aligned, we concatenated our data into a multi-locus dataset with SequenceMatrix 1.8 (Vaidya, Lohman & Meier, 2011) for phylogenetic analysis. The four genes used are elongation factor 1-alpha F1 (*EF1α~~-~~-F1* 1074 bp), elongation factor 1-alpha F2 (*EF1α~~-~~-F2* 434 bp), long-wavelength rhodopsin (*LwRh* 455 bp), and wingless (*WG* 702 bp).

For our phylogenetic analysis, we used ModelFinder (Kalyaanamoorthy et al., 2017) in IQ-TREE (version 1.6.10; Nguyen et al.~~,~~ 2015) to determine the best evolutionary model for each gene. The partitions with the most similar and likely models were merged in IQ-TREE and used to construct a maximum-likelihood phylogeny with *M. turrifex* as the outgroup and 10,000 ultrafast bootstraps (UFboot2; Hoang et al.~~,~~ 2018). Our resulting consensus tree was annotated in FigTree (version 1.4.3; Rambaut~~,~~ 2016) and edited in Adobe Illustrator.

Chemical analysis

Volatile compounds were extracted from workers sampled from lab-maintained colonies of *M. mikromelanos* sp. nov. (n = 6 colonies) and *M. zeteki* (n = 4 colonies). Samples of 4-10 individuals per colony were placed in HPLC grade methanol solvent. Whole ants from the same colony, or trisected ants (head, thorax, gaster), were placed in separate glass vials with 40–100 µL of solvent. Trisections were used to identify where the most abundant compounds were found and whole specimen extractions confirmed the presence of trace compounds detected. Tools used for trisections were rinsed with ethanol, methanol, and pentane between trisection to prevent cross-contamination. Samples were stored at -20 °C until analysis by gas-chromatography mass-spectrometry (GC-MS). Reported compounds were found in at least trace amounts in two or more extracts of workers of the same species.

Samples of extracts were analyzed at the Virginia Military Institute with gas chromatography–mass spectrometry (GC–MS) using a Shimadzu QP-2010 GC–MS equipped with an RTX-5, 30 m × 0.25 mm i.d. column. The carrier gas was helium with a constant flow of 1 ml/min. The temperature program was from 60 to 250 °C changing 10 °C/min and held at the upper temperature for 20 min. The mass spectrometer was operated in EI mode at 70 eV, and scanning was set to 40 to 450 AMU at 1.5 scans/s. Peaks on chromatograms were identified by database

search (NIST Mass Spectral Data base, V.2, US Department of Commerce, Gaithersburg, MD), published literature spectra, and by direct comparison with commercially available authentic samples. We standardized our resulting compounds for comparison. For each sample, ratios from the chromatogram peaks were converted to proportions and visualized in Adobe Illustrator.

Literature Review

We conducted a literature review for all papers referencing *M. zeteki* or *M.* cf. *zeteki* to identify potentially misnamed species. Using the research databases Web of Knowledge (Clarivate Analytics, Massachusetts, United States), antweb.org (California Academy of Sciences, California, United States), hol.osu.edu (C.A. Triplehorn Insect Collection, Ohio, United States), and personal literature collections, we reviewed papers that were found by the search criterion "*Trachymyrmex zeteki*", "*Trachymyrmex* cf. *zeteki*", "*T. zeteki*", "*T.* cf. *zeteki*", "*zeteki*", and "cf. *zeteki*". We then selected articles that included *M.* cf. *zeteki* or *M. zeteki* as their focal research organism and recorded those that reported the deposition of voucher specimens. We disregarded research articles that did not use physical specimens (e.g., data from molecular databases).

Results

Morphometrics

Nearly all measurement means (Welch's) and ranges (Wilcoxon) are different between the two species (Table 2). The synonymized *M. balboai* samples are within the range of *M. zeteki* samples (see Table S4) and are morphologically similar to the *M. zeteki* type specimen. -*M. mikromelanos* sp. nov. is on average smaller than *M. zeteki* except in the case of the frontal lobe index (FLI). Due to non-significant differences, FLI was excluded from analyses of males and gynes. We observed some overlap in the range of measurements for workers and for males between *M. mikromelanos* sp. nov. and *M. zeteki*. In contrast, gynes are very distinct with few overlapping ranges (Table 2).

WORKERS: For our worker partition, all 15 characters were significantly different between species ($p < 0.003$; Table 2). Our NMDS converged on a two-dimensional solution with an acceptable stress level (stress = 0.1288) and the Sheppard plot showed good association around the regression line (non-metric fit $R^2 = 0.983$; linear fit $R^2 = 0.933$; see Fig. S1a). The Resulting NMDS plot shows some overlap between the ellipses, although each species forms a distinct cluster with few outliers (Fig. 1a). The vectors for head width (HW), scape index (SI), and petiole length (PL) showed the most strength and direction in the measurements relative to the NMDS axes (Fig. 1a). Additionally, the type specimens for *M. mikromelanos* sp. nov. and *M. zeteki* plotted within their own ellipses (Fig. 1a). While the *M. mikromelanos* sp. nov. type and paratype specimens fall within the overlap of ellipses for both species, **they remain morphologically distinct (see diagnosis and description)**. For *M. mikromelanos* sp. nov., SI and frontal lobe index (FLI) explain separation from the *M. zeteki* cluster; while HW, eye length (EL), and frontal lobe (FL) explain separation from *M. mikromelanos* sp. nov. for *M. zeteki*. However, PL and waist length (WaL) best explain variation within clusters along the Y axis.

Lastly, the two synonymized *M. balboai* syntype ('cotype') samples fall well within the *M. zeteki* ellipses.

GYNES: For the gyne partition, all but FLI ($p = 0.6110$) were significantly different between species ($p < 0.003$; Table 2). The NMDS converged on a two-dimensional solution with a robust stress level (stress = 0.1119), and the Shepard plot showed a strong association around the regression line with a single outlier (non-metric fit $R^2 = 0.986$; linear fit $R^2 = 0.941$; see Fig. S1b). The NMDS plot showed *M. mikromelanos* sp. nov. and *M. zeteki* each forming distinct clusters with few outliers (Fig. 1b). The *M. mikromelanos* sp. nov. paratype gynes (four specimens highlighted in the figure with x inside the circle) fell well within the *M. mikromelanos* sp. nov. cluster (Fig. 1b). The vectors EL, scape index (SI), and PL showed the most strength in directionality of the measurements relative to the NMDS axes (Fig. 1b).

MALES: For our male partition, all but FLI ($p = 0.0307$) were significantly different between species (Table 2). The NMDS converged on a two-dimensional solution with a robust stress level (stress = 0.1554). The Shepard plot also showed relatively high correlation with the regression line (non-metric fit $R^2 = 0.976$; linear fit $R^2 = 0.886$; see Fig. S1c). The NMDS plot showed *M. mikromelanos* sp. nov. and *M. zeteki* each forming distinct clusters with no outliers. The vectors for PL, mesosoma length (ML), SL, and cephalic index (CI) show the most strength in directionality of the measurements relative to the NMDS axes (Fig. 1c). The paratypes for both males fell well within their species clusters.

Our morphometric analysis shows that *M. mikromelanos* sp. nov. and *M. zeteki* are distinct species while supporting the previous synonymy of *M. balboai* under *M. zeteki* by Weber (1958). Nearly all of the measurements taken are significantly different for all castes. The NMDS plots reflect the overlap of some measurements observed in workers and males while depicting clear separation of measurements observed in gynes.

Behavioral Assay-

The tempo of worker activity differed between the two species (Fig. 1d). A gamma distribution with an inverse link function was the best fit model (Table 3). For our diagnostic analysis of our GLMM see supplementary material (Figs. S2-S4). The gamma inverse model shows that tempo was correlated with species (Table 3, $Pr(>|z|) = 1.150 \times 10^{-02}$) and the variance of the random effect (colony) was not significant (var. $= 7.977 \times 10^{-02}$). This indicates that the variation observed in tempo was associated with species identity rather than with the particular colony of origin. This result provides further support for the delimitation between *M. zeteki* and *M. mikromelanos* sp. nov.

Phylogenetic analysis

Using published data (Nygaard et al., 2016; Solomon et al., 2019) located in GenBank (*M. ~~ycetomoellerius~~ zeteki* genome: GCA_001594055.1) and the *Mycetomoellerius* gene sequences (Dryad DOI: doi:10.5061/dryad.2p7r771; GenBank accession numbers Table S2) we found genetic differences between *M. mikromelanos* sp. nov. and *M. zeteki*, with the former

supported as genetically distinct from the latter by 100% bootstrap support (Fig. 1e). We located scaffolds for four genes (i.e., *EF1α-F1*, *EF1α-F2*, *LwRh*, and *WG*) and found high support for each in the published genome. For the mitochondrial gene *COI*, commonly used for DNA barcoding (Simon et al., 1994), 12 scaffolds were identified in the *M. zeteki* genome and only five had > 95% query cover (Table S3) suggesting the presence of pseudogenes and rendering this marker unreliable (Leite, 2012). Based on the BIC scores, Modelfinder joined *EF1α-F1* + *WG* and *EF1α-F2* + *LwRh* partitions and found the K2P+I and K2P to be the best fit models for those partitions respectively. The samples RMMA090930-09, RMMA050105-29, JSC030826-01, and the genomic scaffold sequences used (GCA_001594055.1) were identified as identical. Our phylogenetic analysis using four genes provided strong support for identifying the Nygaard et al. (2016) genome as belonging to *M. mikromelanos* sp. nov. rather than to *M. zeteki* as reported.

Chemical analysis

We found five farnesene compounds in *M. mikromelanos* sp. nov. and *M. zeteki* workers (**1**) E-β-farnesene, (**2**) (3Z,6E)-α-farnesene, and (**3**) (3E,6E)-α-farnesene, in whole samples and gaster trisections. Farnesenes have been reported before and are presumably localized in the gaster, functioning as trail pheromones (Adams et al., 2012; Figs. 1f, g; Table 4). (3E,6E)-α-farnesene (**3**) is most abundant in *M. mikromelanos* sp. nov., averaging 69.3% of the observed farnesenes. (**1**) and (**2**), are each at less than 23% of the overall abundance in *M. mikromelanos* sp. nov. E-β-farnesene (**1**), is the most abundant (62.2%) in *M. zeteki* with (**2**) at 18.4% and (**3**-~~5~~) with 6.5%.

These results illustrate that unique worker chemical profiles distinguish the two species. Some samples contained dilute concentrations of compounds as seen by the relative abundance (~~f~~Fig. 1f, g). One *M. mikromelanos* sp. nov. colony (CRC170518-08) has a chemical profile similar to *M. zeteki*, with (**1**) 56.9%, (**2**) 33.7%, and (**3**) 9.3%. **While this one colony stands out, all of the colonies of *M. mikromelanos* sp. nov. analyzed are morphologically distinct from *M. zeteki* and fit the description of *M. mikromelanos* sp. nov. (see Taxonomy section).**

Literature Review

We found sixty-three articles that used *M. zeteki* or *M.* cf. *zeteki* under our search criteria (see ~~a~~Appendix ~~t~~Table 1). Twenty-eight articles did not identify the repositories of their voucher specimens, and of these, three articles deposited online sequence vouchers for ant specimens but mentioned no corresponding voucher specimens; nine others deposited symbiont vouchers (two fungal cultivar and seven non-cultivar symbionts). Voucher specimens were deposited in museums around the globe (Appendix Table 1), with the greatest number (fifteen) deposited at the **Smithsonian Institution National Museum of Natural History, United States (USNM)**. The full list of voucher repositories includes: ~~-~~Coleccion Nacional de Referencia Museo de Invertebrados Universidad de Panama (Panama); Smithsonian Tropical Research Institute Panama (Panama); Museu de Zoologia da Universidade de São Paulo (Brazil); Instituto Nacional de Biodiversidad (Costa Rica); Museo de Entomologia de la Universidad del Valle (Colombia); Museo Entomológico Universidad Nacional Agronomía Bogotá (Colombia); Museum at the

Universidad Técnica Particular de Loja (Ecuador); Natural History Museum of Denmark, (Denmark); Zoological Museum of the University of Copenhagen (Denmark); Zoological Museum, University of Puerto Rico (Puerto Rico); and the Smithsonian Institution National Museum of Natural History~~,~~ (United States of America).

Mycetomoellerius mikromelanos sp. nov. Cardenas, Schultz~~,~~ & Adams~~, new species~~

Geographic range: Panama: Colón, Darién, and Panama Province (RMMA & **JLC** specimens).

Label text: Separate labels for each specimen indicated by brackets (e.g., [Label 1] [Label 2]).

HOLOTYPE: Worker, Republic of Panama. [9.16328, -79.74413, Panama: Colón Province, Pipeline Rd, 16E, 62m, 13.v.2017, Cody Raul Cardenas, CRC170513-04] [USNMENT01123723]. Repository: USN**M**.

PARATYPES: 15 Workers, Republic of Panama. ~~-~~Same label data as holotype. Repositories: USNM (3): USNMENT01123726, USNMENT01123727, USNMENT01123728; MZSP (4): OSUC 640618, OSUC 640619, OSUC 640620, OSUC 640621; STRI (5): OSUC 640635, OSUC 640636, OSUC 640637, OSUC 640638, OSUC 640639; OSUC (3): OSUC 640606, OSUC 640607, OSUC 640608.

PARATYPES: 10 Gynes, Republic of Panama. Same label data as holotype. Repositories: USNM (4): USNMENT01123724, USNMENT01123729, USNMENT01123730, USNMENT01123731; **MZSP (1): OSUC 640622, OSUC 640623, OSUC 640624**; STRI (3): OSUC 640640, OSUC 640641, OSUC 640642; OSUC (1): OSUC 640609.

PARATYPES: 7 Males, Republic of Panama. Same label data as holotype. Repositories: USNM (4): USNMENT01123725, USNMENT01123732, USNMENT01129733, USNMENT01129734; MZSP (1): OSUC 640625; STRI (1): OSUC 640643; OSUC (1): OSUC 640610.

HOLOTYPE/PARATYPE Colony Code: CRC170513-04.

Additional material examined

Workers N=13: USNM: 12 specimens sharing label data [PANAMA: Pipeline RD, La Seda River; 79.736°'W 9.1529°'N; 28 v 2010;] [Henrick H. De Fine Licht; nest series; river bank; underground' HDFL28052010-4 ch1] [*Trachymyrmex zeteki*] [Check cryo] [DO NOT REMOVE SI DB Reference Not a property tag T. Schultz, NMNH]: **USNMENT00752565**, **USNMENT00752578**, **USNMENT00752579**; Sharing label data [PANAMA: Pipeline Rd, La Seda River; 79.736° W, 9.1529°'N; 28 v 2010;] [Henrik H. De Fine Lichtl nest series; river bank; underground HDFL28052010-5] [*Trachymyrmex zeteki*] [~~S~~see cyro collections] [DO NOT REMOVE SI DB Reference Not a property tag T. Schultz, NMNH]: **USNMENT00752574**, **USNMENT00752580**, **USNMENT00752581**, **USNMENT00752582**; Sharing label data [PANAMA: Pipeline Road, 2km past Limbo RiverL 12v2010] [Henrik H. De Fine Licht; nest series; river bank; underround HDFL120502010-14] [*Trachymyrmex zeteki*] [~~S~~see also cryo

collections] [DO NOT REMOVE SI DB Reference Not a property tag T. Schultz, NMNH]: **USNMENT00752565**, **USNMENT00752578 (1 pin/-2 specimens)**, **USNMENT00752579**. JTLC: 1 specimen [PANAMA, Darién: 5 km S Platanilla 8.78105 -78-.41251 ±20m 160m, 20an2015 J. Longino#9082] [2nd growth veg. stream edge nest in clay bank] [**CASENT0633645**].

Males N=3: USNM: 3 specimens sharing label data [PANAMA: Pipeline Road, 2km past Limbo River 12v2010] [Henrik H. De Fine Licht; nest series; river bank; underground HDFL120502010-14] [*Trachymyrmex zeteki*] [Ssee also cryo collections] [DO NOT REMOVE SI DB Reference Not a property tag T. Schultz, NMNH]: USNMENT00752576, USNMENT00752578 (1 pin/-2 specimens).

Note: A name previously applied to this species, *Trachymyrmex fovater*, was incorrectly electronically published in a conference poster format and is therefore unavailable (Cardenas et al., 2016). This name is unavailable because (i) the date of the publication was not indicated and (ii) the name was not registered in the Official Register of Zoological Nomenclature (ICZN, 1999). We hereby describe *Mycetomoellerius mikromelanos* sp. nov. (LSID: urn:lsid:zoobank.org:act:B6BABA13-708F-44D8-AD2C-F4D5B8FB03E8), a name more appropriate for this species (see Etymology) and provide a complete diagnosis and description of this new species.

Diagnosis: Measurements for all castes are in Table 12. We found characters that reliably separate *M. mikromelanos* *sp. n.* from *M. zeteki*. However, due to the variability of worker castes, intermediate character states occur in some individuals. The following characters are those most useful for diagnosis. Workers: 1) cuticle coloration dark-ferrugineous (Figs. 2a, b); 2) overall integument bearing granulose irrorate sculpturing (Figs. 2a, b); 3) frontal lobe with crenate margins and weak anterolateral spine (Fig. 2b); 4) hooked spatulate bi-colored setae medial to frontal carinae on disc of head capsule (Fig. 2b); 5) scape surpassing occipital corners when lodged in antennal scrobe (Fig. 2b); 6) convex margin of the compound eye extending past the lateral border of the head by more than half of its visible diameter in full-face view (Fig. 2b). Gynes: 1) cuticle coloration dark-ferrugineous (Figs. 2c, d); 2) supraocular spine superior to compound eye by more than or equal to eye's length (Fig. 2c); 3) small arcuate ridge superior to and reaching anterior central ocellus, with its terminal ends directed posterolaterally (Fig. 2c); 4) lateral ocelli partially obscured in full-face view (Fig. 2c); 5) mesoscutum with random-reticulate sculpturing (Fig. 2e); 6) wings bicolored, venation ferrugineous-brown (Figs.1e, f); 7) hindwing with 7-9 hamuli (Fig. 2f). Males: 1) bicolored; head and mesosoma ferrugineous-brown; metasoma dark testaceous-orange (Fig. 3a); 2) complete carinate-rugulose sculpturing of posterior head capsule, arranged nearly perpendicular to the longitudinal axis of the head (Fig. 3a); inferior to frontal lobes, sculpturing sparsely carinate and finely reticulate (Figs. 3a; Fig. 14); 3) mandibles distinctly smaller compared to *M. zeteki*; 4) corners of medial clypeal emargination rounded (Fig. 3b); 5) ocelli smaller relative to *M. zeteki* in full-face view, occipital corners of head capsule visible (Fig. 3b); 6) propodeal spines wider at base than long (Fig. 3a).

WORKER (description): Pilosity and color: older workers dark-ferrugineous; young workers ferrugineous-orange. Integument with granulose irrorate sculpturing; **white cuticular bacterial bloom variably present among workers** (Fig. 2a). Pilosity strongly bicolored, terminating with light coloration when spatulate, otherwise curved, appressed, and simple. Head: in full-face view, head broader than long, with weakly granulose sculpturing. Palpal formula 4,2. Mandible feebly sinuous, with 6-9 denticles. Median margin of clypeus impressed, lateral-most corners of impression distinctly angulate. Frons with bi-colored setae. Originating from mandibular insertion, preocular carinae subparallel, reaching occipital corners, terminated by a stout multituberculate tumulus directed posterolaterally. Frontal lobe semicircular, with crenate margins and weak anterolateral spine (Fig. 2b). **Frontal carinae subparallel, extending from frontal lobes to vertex margins**. Each eye with 6-7 facets across width. Convex margin of compound eye extending past the lateral border of the head by more than half of its visible diameter in full-face view (Fig. 2b). **Frontal carinae extending from posterior margins of frontal lobes to occipital corners**, joining the subparallel preocular carinae to form antennal scrobes. Antenna with 11 segments. Scape wide proximally, weakly tapering before thickening sub-distally, narrowing at apex; when lodged in antennal scrobe, scape surpasses occipital corner. Disc of head capsule bears spatulate and bi-colored setae (Fig. 2b). Supraocular projection stout, multituberculate. Vertex impression shallow and narrow, but variable. Mesosoma: sparse rugulose sculpturing, most mesosomal sclerites with granulate sculpturing. Pronotum with median pronotal tubercle, superior pair of pronotal spines that project anterolaterally, and inferior pair of pronotal spines that project anteroventrally. **In most cases, median pronotal spine projects as far or farther than lateral pronotal spines.** Dorsum of propodeum, in lateral view, has distinct, tuberculate carinae at anterior base of propodeal spines. Carinula bearing variable number of tubercles along lateral face of propodeum and superior margin of metapleural gland bulla, occurring from spiracle to propodeal lobes. Coxae II and III have spatulate setae on parallel carinae dorsolaterally. Coxa I with subtle superior impression on its anterior margin. In lateral view, coxa I is longest and coxa II is shortest. Metasoma: petiole granulate. **Petiolar node variable in number of spines, typically two to three**, along carinae. Carinae almost reaching posterior margin, weakly turning mesad anteriorly but not touching. Lateral posterior margin weakly convex; dorsal posterior margin weakly concave and subtly crenulate. In dorsal view, lateral margins weakly convex, with mostly symmetrical tubercles. Ventral petiolar carinula converge posteriorly to subpetiolar process. Postpetiole with spatulate setae dorsally, pair of simple setae ventrally, and intermittent dorsal tubercles with posterior impressions. Postpetiole broader than long dorsally. Posterior margin of postpetiole impressed in lateral view and weakly crenulate. Posterior margin of petiole in dorsal view flat medially, with medial impressions on lateral margins. In dorsal view, lateral margins rounded anteriorly and impressed posteriorly. Gaster somewhat triangular when viewed anteriorly. Laterally, gaster mostly round, with weakly reticulate sculpturing. Anterior setae of tergite and sternite spatulate. First gastral tergite has crenate posterolateral corners that surpass thin shiny margin between tergites I and II. Posterior margin of first tergite with subtly curved, simple setae. Tergites and

sternites two to four with simple setae that become gradually finer and lighter posteriorly. Terminal tergites and sternites with dense, lightly colored setae.

GYNE (description): Gynes share many characters with workers. Pilosity and color: ~~Y~~young gynes uniform ferrugineous-orange color, increasingly dark-ferrugineous with age (Figs. 2c, d). Dark spatulate curved setae, bi-colored setae occur on mesosoma and metasoma. Head: in full-face view, head longer than broad. Setae of head capsule dark, curved, appressed, and simple; setae spatulate mesad of frontal carinae. Mandibles with 6-8 denticles. Frons between frontal carinae with rugose sculpturing. Minute tubercles posterior to clypeus and anterior to frontal lobes-. Frontal lobe margins crenulate, with carinae interior and parallel to margins; anterolateral margin with reduced spine. Face of frontal lobes weakly rugulose. In full-face view, more than three quarters of the anterior lateral margin of compound eye surpassing lateral margin of head capsule. Antennal scapes wide proximally and tapering slightly before thickening subdistally. Supraocular spine separated from compound eye by as much or more than eye's length (i.e., EL = 0.27 mm, distance to supraocular spine = 0.31 mm). Vertexal carinae extending from ocelli to frontal carinae. Small arcuate ridge touches posterior margin of ocellus superior to anterior ocellus; its terminal ends directed posteriorly. Vertex variably impressed, but generally shallow and narrow. Mesosoma: curved, appressed setae on mesoscutum and mesoscutellar disc; spatulate setae on other mesosomal sclerites. Confused-rugulose sculpturing on mesosomal sclerites, except for mesoscutum and mesoscutellar disc, which have random-reticulate sculpturing. Medial spine of pronotum stout, projecting anteriorly; superior lateral pronotal spine projecting anterolaterally, inferior lateral pronotal spine projecting ventrolaterally, flattened laterally. Mesoscutellar disc with two small spines that project posteriorly. Axilla hides scutoscutellar sulcus. Katepisternum and anepisternum suture embossed with strigate sculpturing. Inferior margin of anepisternum crenulate. Propodeal declivity nearly vertical. Coxa I with dark curved setae, and smaller dense curved setae throughout, with weak asperous sculpturing on lateral face. Coxa II with spatulate setae along parallel carinae, with a row of thick, dark, curved setae on posterior side in lateral view; coxa II and coxa III have confused rugulose sculpturing lateral to carinae. Coxa III has spatulate setae along carinae, and simple setae throughout. In lateral view, coxa I longest, coxa II shortest. Wings: tegula triangular and weakly impressed on its face. Axillary sclerite well developed, covered with setae, flattened along distal margin. Forewing with five cells (Fig. 2f). Wing venation ferrugineous-brown, front and hind wings tinted smoky gray, more so anteriorly and less so posteriorly. Length of r-rs vein greater than half the length of the section of Rs vein between r-rs and M veins (Fig. 2f). Hindwing with 7-9 hamuli (Figs. 2d, f). Metasoma: p~~P~~etiole with weakly appressed setae. Dorsal carinae of petiole with spines that are parallel and touch posterior margins of petiole. Dorsal carinae directed medioanteriorly but not joining. Ventral petiolar carinulae converging posteriorly on subpetiolar process. Postpetiolar dorsum with distinct tubercles, lightly impressed medially. Posteriorly in dorsal view, postpetiole bears two impressions on posterolateral margins. Postpetiole with subtle medial impression on posterior margin. Gaster with reticulate sculpturing. First gastral tergite has simple setae. In lateral view, first sternite and first gastral tergite have

confused-costulate sculpturing. Gastral tergites I-IV have crenulate carinae just bordering narrow shiny posterior margin. Terminal tergites have dense, lightly colored setae surrounded by dark setae; setae becoming less appressed towards terminal tergites and sternites.

MALE (description): Pilosity and color: mature males bicolored, head and mesosoma testaceous-orange and dark-ferrugineous, abdomen testaceous-orange (Fig. 3a). Integument with generally weak to effaced rugulose sculpturing (Fig. 3a). Head: capsule in full-face view wider than long (Fig. 3b). Head capsule sculpturing carinate-rugose, sparsely carinate and finely reticulate inferior and lateral to frontal lobes. Striate sculpture of head capsule in profile arranged nearly perpendicular to the longitudinal axis of the head (Figs. 3a; ~~see also Fig.~~ S5). Mandibles elongate-triangular, feebly sinuous, with lightly colored appressed setae. Entire apical masticatory margin darker than rest of mandible. Prominent apical t~~ee~~ooth with variably sized proximate teeth denticulate, with 4-6 denticles. External margin feebly sinuate, with appressed setae. Clypeus evenly rounded and weakly sculptured except narrow shiny anterior margin. Frons bulbous with weak to effaced carinate sculpturing across its entirety, forming two small mounds inferior to the frontal lobes. In lateral view, preocular carina continuing along inner margin of eye variably extending posterad. Frontal lobes deeply impressed medially, with smooth margins. Neck of scape and basal condyle visible (Fig. 3b). Antennae with 13 segments; scape wide proximally, gently narrowing to apex, covered with very fine, lightly colored setae pressed against cuticle (Fig. 3b). In full-face view, lateral ocelli prominent and separated by a shallow vertexal impression (Fig. 3b). Supraocular projection absent or weak, when present directed posteriorly and near ocellus in full-face view. Mesosoma: sculpturing weak to effaced carinulate-rugulous throughout, finely reticulate where carinulate-rugulous sculpturing absent. Setae appressed throughout. Pronotum with small lateral spines that project anterolaterally. Forward-projecting median pronotal tubercle near mesoscutum and pronotal suture. Median pronotal tubercle varying from clearly visible to greatly reduced, best seen laterally. At inferior corner of pronotum, anterior to coxa I, **carinae** without or with~~bear~~ an **extremely reduced** inferior spine~~or absent inferior spine~~. Mesoscutum rounded and bulbous anteriorly, bulging over pronotal-mesoscutal suture. Mesoscutellar disc with two very small, posteriorly projecting spines. Propodeum with small posterior spines that are wider, or as wide at the base as long, projecting posterolaterally (~~f~~Fig. 3a). Coxae mostly covered with light-colored setae, coxa I with carinulate-rugulose sculpturing. Coxa II with dark prominent setae posteriorly, near trochanter. Coxa I longer than coxa III, coxa II shortest. Wings: forewing weakly bicolored with minute pilosity and five cells. M+Cu exceeds half length of 1A after the cu-a proximally. Length of r-rs vein greater than half length of section of Rs vein between r-rs and M veins. Hindwing with 6-8 hamuli. Metasoma: petiole weakly costulate in sculpturing, with curved setae dorsally. Petiolar node rounded, with spiracle anterior to center of node. Dorsally, lateral margins impressed, with anterior spine larger. In lateral view, postpetiole nearly rectangular. Dorsally, posterior margin shallowly impressed. Gaster with fine reticulate sculpturing. All setae of first gastral tergite appressed; those on tergites 2-5, weakly appressed along posterior margins. Setae on sternites

follow the same pattern as those on tergites. Pygostyle and genital opening densely covered with lightly colored setae.

Etymology

"Mikromelanos" is a singular, masculine adjective, compounded from the Greek **μικρός** (mikrós), meaning "small," and **μελανός** (melanós), meaning "black" or "dark." This etymology highlights the authors' colloquial use of "big red" to describe the larger red queens of *M. zeteki* and "little black" to describe the smaller darker queens of *M.* ***mikromelanos***.

Comments

Although *M. mikromelanos* shares many similarities with *M. zeteki* (Fig. 2-5; Weber, 1940, 1958b; Mayhé-Nunes ~~and~~& Brandão, 2007), certain key characters allow us to easily distinguish the two species with a 20X loupe in the field. These key characters in *M. mikromelanos* are (i) the worker scapes extend past the occipital corners of the head capsule (extending only to the occipital corners in *M. zeteki*), (ii) gyne wing venation is ferrugineous-brown in *M. mikromelanos* and testaceous-orange in *M. zeteki*, (iii) gynes of *M. mikromelanos* are typically smaller and a dark reddish brown, where *M. zeteki* gynes are larger and a bright reddish color, (iv) males are bi-colored, dark-ferruginous and testaceous-orange (uniform, testaceous-orange in *M. zeteki*), and (v) in general, all castes of *M. mikromelanos* are smaller than those of *M. zeteki*. Distinguishing between the gynes of *M. mikromelanos* and *M. zeteki,* however, requires a microscope. Aside from size, it is most informative to look at sculpturing of the mesoscutum under a microscope: *M. mikromelanos* gynes have random reticulate sculpturing on the mesoscutum whereas *M. zeteki* have parallel sculpturing. In addition to color differences, males of the two species can be differentiated by the integumental sculpture near the eye. In the male of *M. mikromelanos*, in lateral view, the striations follow the contours of the ventroposterior borders of the eye (Figs. 3a, ~~&~~ S5), whereas in *M. zeteki* they fan outward from the ventroposterior corner of the head and are interrupted by the borders of the eye and the preocular carina, where they end (Figs. 5, ~~&~~ S8). A complete list of measurements is provided in the supplementary material.

Biology

Mycetomoellerius mikromelanos is the most common 'funnel *Mycetomoellerius*' found on Pipeline Road, near Gamboa, Panama. Young queens establish their nests from the start of the rainy season (May) into July. They nest in vertical clay embankments with entrances shaped like funnels (i.e., auricles) with flared margins (Mueller & Wcislo, 1998; Pérez-Ortega et al., 2010). Colonies are often tucked under roots or overhangs and occur in high densities (~5 cm apart) along creeks or are isolated in the forest at the base of trees. Colonies of *M. mikromelanos* have up to five vertically arranged chambers with single vertical tunnels between them. We removed the auricles from 16 nests and 15 were rebuilt to roughly the same size within seven days, suggesting the funnel structure may have some kind of biological function (Figs. S6, ~~&~~ S7; also

see Mueller ~~and~~& Wcislo, 1998; Schultz et al., 2002; Pérez-Ortega et al., 2010; Helms et al., 2014).

A variety of organisms exploit the resources of *M. mikromelanos* (e.g., fungal garden, shelter, brood). *Megalomyrmex adamsae* Longino 2010, a rare obligate social parasite (1-6% parasitism rate), forages on the host garden and brood and never leaves the nest of *M. mikromelanos* (Adams et al., 2012b). *Escovopsis* Muchovej & Della Lucia 1990, a micro-filamentous fungal parasite, is maintained at low levels due to specialized grooming behaviors used by workers of *M. mikromelanos* (Currie, Mueller & Malloch, 1999; Currie et al., 2003; Little et al., 2003, 2006). Other fungi such as *Trichoderma* Persoon 1801 threaten the health of the garden and are managed by the ants (Currie et al., 2003; Little et al., 2006). There are also six Diapriinae morphospecies exploiting *M. mikromelanos*, but little natural history has been reported for these associations (but see Pérez-Ortega et al., 2010). Diapriinae parasitoid wasps infiltrate nests and parasitize host larvae, turning them black as the wasps develop internally. We found that mature wasp pupae can be prompted to eclose when disturbed or picked up and male *Acanthopria* sp. Ashmead 1895 tend to naturally emerge before *Acanthopria* females in captive colonies (ca. 10 days). We also found that *Mimopriella* sp. Masner & Garcia 2002 can take up to six months to complete development in a laboratory-maintained colony. The mechanism behind this unusually slow growth is unknown. These symbionts highlight the known diversity of a species network that is reliant on *M. mikromelanos* for survival.

Mycetomoellerius zeteki (Weber~~,~~ 1940)

Geographic range: Colombia, Costa Rica, Ecuador, Panama (Mayhé-Nunes & Brandão, 2007)

Label text: Separate labels for each specimen indicated by brackets (e.g., [Label 1] [Label 2]).

LECTOTYPE (here designated): Worker~~:~~, [Barro Colorado. CANAL ZONE No. 756 N.A.Weber 1938] [*Trachymyrmex zeteki* Weber COTYPE] [USNMENT01129855]. Repository: MCZ.

PARALECTOTYPE (examined): Worker.~~:~~ [Barro Colo. I. Canal Zone No.756 NA Weber 1938] [*M.*C.Z. CoType 25619] [*T. zeteki* Weber Cotypes] [Harbor Islands Insect Database] [MCZ-ENT 00025619]. Repository: MCZ.

Additional material examined

Workers **N = 24**: MCZ: ~~(pin,~~ **1** specimen~~)~~ [Barro Colo. I. Canal Zone No856 NAWeber 1938 walking at 9 p*M.* Snyder-Molino 0-4.] [762 1 worker USNM]; ~~(pin,~~ **2** specimens~~)~~: [Barro Colo. I. Canal Zone No. 759 NA Weber 1938] [*T. balboai* Weber Cotypes]. **NHMB:** ~~(pin,~~ **1** specimen~~)~~ [Barro Colo. I C.Z. 3441 NAWeber] [*Trachymyrmex zeteki* Weber] [17.vi.56 3441] [ANTWEB CASENT 0912534]; NOTE: The NHMB pin bears a "type" label, but we assume it to be erroneous because the specimen was collected in 1956 and therefore cannot be part of Weber's 1938 *M. zeteki* syntype series. **[Barro Colo. I C.Z. 3441 NAWeber] [*Mycetomoellerius zeteki* Weber] [ANTWEB CASENT 0912534]**; USNM: **3** specimens sharing these label data

[PANAMA: Pipeline Rd; 19 v 2010; Henrik H. De Fine Licht; nest series; river bank; underground; HDFL1952010-8] [see also cyro collections] [*Trachymyrmex* sp's] [DO NOT REMOVE SI DB Reference Not a property tag T. Schultz, NMNH] USNMENT00752570 (1 pin/-2 specimens), USNMENT00752572 ~~(pin 1 specimen)~~. **16** specimens sharing these label data: [9.1624,-79.74802, PANAMA: Colón, Pipeline Rd, Bird Plot 4E19N, 70m, 29.vi.2010, Rachelle MM Adams, RMMA100629-15] [Formicidae Myrmicinae *Trachymyrmex zeteki*, Weber 1940, det. Cardenas, CR., 2018]. Repositories: USNM (4): USNMENT01129711, USNMENT01123714, USNMENT01123715, USNMENT01123716; MZSP (4): OSUC 640611, OSUC 640612, OSUC 640613, OSUC 640614; STRI (5): OSUC 640626, OSUC 640627, OSUC 640628, OSUC 640629, OSUC 640630; OSUC (3): OSUC 640601, OSUC 640602, OSUC 640603.

Gynes **N = 9:** Sharing these label data~~:~~ [9.1624,-79.74802, PANAMA: Colón, Pipeline Rd, Bird Plot 4E19N, 70m, 29.vi.2010, Rachelle MM Adams, RMMA100629-15] [Formicidae Myrmicinae *Trachymyrmex zeteki*, Weber 1940, det. Cardenas, CR., 2018]. Repositories: USNM (4): USNMENT01123712, USNMENT01123717, USNMENT01123718, USNMENT01123719; MZSP (2): OSUC 640615, OSUC 640616; STRI (2): OSUC 640633, OSUC 640634; OSUC (1) OSUC 640604.

Males **N = 11**: USNM: 3 specimens sharing these label data [PANAMA: Pipeline Rd; 19 v 2010; Henrik H. De Fine Licht; nest series; river bank; underground; HDFL1952010-8] [see also cyro collections] [*Trachymyrmex* sp's] [DO NOT REMOVE SI DB Reference Not a property tag T. Schultz, NMNH]: USNMENT00752568, ~~and~~ USNMENT00752570 (1 pin/-2 specimens). Sharing these label data~~:~~ [9.1624,-79.74802, PANAMA: Colón, Pipeline Rd, Bird Plot 4E19N, 70m, 29.vi.2010, Rachelle MM Adams, RMMA100629-15] [Formicidae Myrmicinae *Trachymyrmex zeteki*, Weber 1940, det. Cardenas, CR., 2018]. Repositories: USNM (4): USNMENT01123713; USNMENT01123720; USNMENT01123721; USNMENT01123722; MZSP (1): OSUC 640617; STRI (2): OSUC 640633, OSUC 640634; OSUC (1): OSUC 640605.

Mycetomoellerius zeteki was originally described by Weber (1940) as *Trachymyrmex zeteki* from an accidental collection in dense shade on a slope near the lab on Barro Colorado Island, Panama Canal Zone (Weber, 1940; Mayhé-Nunes & Brandão, 2007). In the same article Weber followed his description of *T. zeteki* with a description of *T. balboai* (Weber, 1940). These descriptions were based on small series of workers from single collections. Weber noted similarities between the two species in his original descriptions. According to Weber, *T. zeteki* was distinctly smaller than *T. balboai*, paler in appearance, and the relative proportions of the thoracic spines differed. The character states that Weber used to differentiate the two species were later understood to represent variation within a single species and *T. balboai* was synonymized with *M. zeteki* (Weber, 1958b). In Mayhé-Nunes and Brandão's (2007) revision of *Mycetomoellerius*, *M. zeteki* was placed in the "Jamaicensis group~~,~~", a subset of the "Iheringi group~~.~~". Distinct characteristics of the Jamaicensis group are the open antennal scrobes arising from the subparallel preocular and frontal carinae (Mayhé-Nunes & Brandão, 2007), a character cited by Solomon et al. (2019) as

applying to the entire genus *Mycetomoellerius*. Here we provide a diagnosis of all castes and describe the gyne wing venation and the males of *M. zeteki*. For complete descriptions of worker and gynes of *M. zeteki*, see Weber (1940, 1958b) and Mayhé-Nunes and Brandão (2007).

Diagnosis: Measurements for all castes are found in Table 12. Certain characters are useful for separating *M. zeteki* from *M. mikromelanos* sp. nov. However, due to the variability of the worker castes, intermediate character states occur in some individuals. The following characters are most useful. Worker~~s~~: 1) cuticle ferrugineous (Figs. 4a, b; dark-ferrugineous in *M. mikromelanos*); 2) integumental sculpture weakly irrorate (Figs. 4a, b; granulose irrorate sculpturing in *M. mikromelanos*); 3) frontal lobe with weakly crenulate margins and distinct anterolateral spine (Fig. 4b; crenulations present and spines lacking in *M. mikromelanos*); 4) disc of head capsule between frontal carinae mostly lacking strongly hooked spatulate bi-colored setae (Fig. 4b; present *in M. mikromelanos*); 5) scape of antenna reaching occipital corners when lodged in antennal scrobe (Fig. 4b; surpassing occipital corners in *M. mikromelanos*); 6) convex margin of the compound eye extending past lateral border of head capsule by less than half of the eye area in full-face view (Fig. 4b; extending by more than half in *M. mikromelanos*). Gyne: 1) cuticle coloration ferrugineous (Figs. 4c, d; dark-ferrugineous in *M. mikromelanos*); 2) supraocular tubercle separated from compound eye by a distance less than or equal to the eye length (Fig. 4c; more than or equal to eye's length in *M. mikromelanos*); 3) small arcuate ridge superior to anterior ocellus with terminal ends directed anterolaterally (Fig. 4c; directed posterolaterally in *M. mikromelanos*); 4) lateral ocelli conspicuous in full-face view (Fig. 4c; partially obscured in *M. mikromelanos*); 5) mesosoma with sparse carinate sculpturing; mesoscutum with parallel-costulate sculpturing (Fig. 4e; random-reticulate in *M. mikromelanos*); 6) wing venation testaceous-orange brown (Fig. 4f; wings weakly ferrugineous-brown in *M. mikromelanos*); 7) hindwing with 5-8 hamuli (Figs. 4e, f; 7-9 in *M. mikromelanos*). Male: 1) coloration mostly uniform testaceous-orange (Fig. 5a; bicolored, head and mesosoma ferrugineous--brown with metasoma dark testaceous-orange in *M. mikromelanos*); 2) striations on head capsule fanning outward from ventroposterior corner of head, ending at the compound eye and preocular carina (Figs. 5a; ~~Fig.~~ 17; striations perpendicular to longitudinal axis in *M. mikromelanos*); sculpture prominent on posterior head capsule, minute to absent anteriorly (Fig. 5a; nearly complete sculpturing of head capsule in *M. mikromelanos*); 3) mandibles larger compared to those of *M. mikromelanos*; 4) corners of clypeal emargination slightly angled (Fig. 5b; rounded in *M. mikromelanos*); 5) in full-face view; occipital corners of head capsule partially obscured by large ocelli (Fig. 5b; visible in *M. mikromelanos*); 6) propodeal spines longer than width of spine at base (Fig. 5a; wider at base than long in *M. mikromelanos*).

GYNE (wing description): face of tegula triangular, slightly impressed. Axillary sclerite covered with setae, flattened along its distal margin. Hindwing with 5-8 hamuli (Figs. 4d, f). Forewing with five cells, wing venation testaceous-orange/brown, wings lightly tinted smoky gray, only slightly more so anteriorly than posteriorly (Figs. 4d, f). Length of r-rs vein less than half the length of section of Rs vein between r-rs and M veins (Fig. 4f).

MALE (description): Pilosity and color: coloration light, mostly uniform, testaceous-orange color (Fig. 5a). -Integument generally weak to effaced carinulate-rugulose sculpturing. Head: capsule in full-face view wider than long (Fig. 5b). Sculpture carinulate-rugulose lateral and posterior to the frontal lobes, otherwise finely reticulate. Sculpture reduced posterior to median ocelli and in median portion of vertex. Striations on head capsule fanning outward from ventroposterior corner of head, ending at the compound eye and preocular carina (Figs. 5; ~~Fig.~~ 17). Mandibles elongate-triangular, feebly sinuous, with lightly colored setae. Entire apical masticatory margin distinctly darker than rest of mandible, with 5-7 teeth. Apical t~~ee~~ooth prominent with proximate teeth variably dentate to denticulate. External margin feebly sinuate, with fine appressed setae. Clypeal margin somewhat shiny and not evenly rounded, forming a slight angle near clypeal emargination. Frons mostly smooth, somewhat bulbous, with carinulate-rugulose sculpturing forming two small mounds superior to clypeal margin and inferior to the frontal lobes. In lateral view, preocular carina continuing along inner margin of eye, above the eye continuing to variably extending posterad. Frontal lobes lightly impressed medially with smooth margins. Neck of scape and basal condyle visible. Antennae with 13 segments; scape covered in fine and intermittent dark setae, wide proximally, gently tapering before widening sub-distally to apex (Fig. 5b). Preocular carina originating near mandibular insertion, continuing along inner margin of eye, finally curving inward towards, but not reaching, the ocelli. In full-face view ocelli large and distinct, lateral ocelli prominent, **forming vertexal impression**. Supraocular projection directed posteriorly, visible in full-face view. Mesosoma: ~~S~~sculpture carinulate-rugulous throughout, weakly reticulate when carinulate-rugulous sculpture absent. Mostly appressed setae throughout. Pronotum with small lateral spines that project anteriorly; minute spines occurs medially along anepisternum pronotal suture. Anterior of coxa I with an extremely reduced spine on carina on inferior corner of pronotum. Mesoscutum rounded and bulbous anteriorly, bulging over pronotal-mesoscutal suture. Axilla hide part of scutoscutellar suture in lateral view. Mesoscutellar disc has two posteriorly projecting spines. Propodeal spines longer than width of base and projecting posteriorly (Fig~~,~~. 5a). Declivity of propodeum nearly vertical. Coxae covered mostly with lightly colored setae, with weak carinulate sculpture. Coxa I with a few dark setae anteriorly, and coxa II with dark prominent setae positioned posteriorly near trochanter. Length of coxa III equal to or longer then coxa I. Wings: Forewing weakly bicolored and covered with minute pilosity, possessing five cells. M+Cu less than half length of 1A after cu-a proximally. Length of r-rs vein less than half the length of section of Rs vein, between r-rs and M veins. Hindwing with 4-7 hamuli. Metasoma: ~~P~~petiole somewhat costulate in sculpturing, with curved setae dorsally. Petiolar node rounded. In profile, spiracle present medially at the anterior margin. In dorsal view, anterolateral tumuli flanking a flattened medial projection. In lateral view, postpetiole somewhat square, with a shallow posterior impression. Posterior ventral side of the postpetiole with setae that may vary in length from minute to almost as long as postpetiole. Gaster sculpturing finely reticulate. All setae of first gastral tergite sparse and appressed, setae on tergites 2-5 also sparse and appressed, with curved dark setae along the posterior margins. Sternite setae follow the same pattern as those on tergites. Pygostyle and genital opening covered with lightly colored setae.

Comments

A specimen of *M. zeteki* deposited at the Natural History Museum, Basel, Switzerland bears a "cotype" label in error. The data label reads as follows '[Barro Colo. I C.Z. 3441 NAWeber] [17.vi.56 3441] [*Trachymyrmex zeteki* Weber] [ANTWEBCASENT0912534] [type].'. It is not possible that this specimen, collected in 1956, 18 years after the *M. zeteki* type series was collected, is a type specimen of that species. While this specimen could be part of the material examined for Weber's 1958 *balboai-zeteki* synonymy, no repositories were mentioned (Weber, 1958b). This specimen was not treated as a syntype for this study. For a complete description of the workers and gyne of *M. zeteki*, see Mayhé-Nunes and Brandão (2007). Certain key characters allow us to easily distinguish *M. zeteki* from *M. mikromelanos* with a 20X loupe in the field. For *M. zeteki* these characters are (i) in workers of *M. zeteki*, the scapes reach the occipital corners of the head capsule but do not extend past them, whereas in *M. mikromelanos*, they extend past the head capsule when lodged in the antennal scrobe, (ii) the gynes of *M. zeteki* are comparatively larger than those of *M. mikromelanos* and are typically bright reddish in color whereas *M. mikromelanos* are generally a darker reddish brown, (iii) gyne wing venation is testaceous-orange in *M. zeteki* and ferrugineous-brown in *M. mikromelanos*, (iv) males are uniform in color and testaceous-orange in *M. zeteki* and bicolored dark-ferrugineous and testaceous-orange in *M. mikromelanos*, and (v) in general all castes of *M. zeteki* are larger than *M. mikromelanos*. It is necessary to note that workers from incipient colonies of *M. zeteki* often resemble workers of *M. mikromelanos*. A complete list of measurements can be found in the supplementary material.

Biology

Most reports of *M. zeteki* are most likely accounts of *M. mikromelanos* (Appendix Table 1). *Mycetomoellerius zeteki* is rare relative to *M. mikromelanos* in the Canal Zone near Gamboa, Panama. For example, we only located two colonies of *M. zeteki* near the type locality on Barro Colorado Island, and one colony at El Llano ca. 40 km east of the canal. On the mainland we have found mixed sites of both species and a single creek with only *M. zeteki* present (Rio Mendoza, ca. 1 km North of Rio La Seda), but when the two species occur together, *M. zeteki* always occurs at comparably lower densities. *Mycetomoellerius zeteki* and *M. mikromelanos* are similar morphologically and biologically and this has led to confusion between these sister species. In both species, young queens establish their nests from the start of the rainy season (around May) into July. Nests can be found on the same clay embankments with indistinguishable auricles with up to five chambers. In the five mature *M. zeteki* nests we excavated, each had two tunnels connecting each chamber. M. mikromelanus had one tunnel connecting the chambers. There are likely other architectural differences, such as volume and internal auricle shape, but more colonies of *M. zeteki* need to be examined.

Mycetomoellerius zeteki and *M. mikromelanos* also have a similar range of symbionts. *Megalomyrmex adamsae* associates with *M. zeteki*, foraging on host garden and brood, and never leaves the host nest (Adams et al., 2012b). An *Escovopsis* fungal parasite attacks the fungal garden. Garden maintenance behavior also appears similar as *M. zeteki* forms infrabuccal pellet

piles like *M. mikromelanos* (Little et al., 2003). We have documented the first Diapriidae wasp parasitizing the brood of *M. zeteki*. In a laboratory colony (CRC170519-01), we observed a male wasp of *Mimopriella* sp. ~~Masner and Garcéa (2002)~~ emerge on May 19th, 2017, and a female 10 days later. The live colony had characteristically black larvae when collected. While some natural history has been documented, there is still much more to be discovered about the symbionts, nest architecture, and general biology of *M. zeteki.*

Discussion

Based on multiple lines of evidence, we have shown that the new species *M. mikromelanos* is a well-studied cryptic species that has been confused with *M. zeteki* for decades. We accomplished this by examining morphology and morphometrics of all castes, analyzing the behavior of workers, comparing worker volatile compounds, and comparing DNA sequence data. Interestingly, we also determined that the published genome (Nygaard et al., 2016) belongs to the newly described species *M. mikromelanos.* Our results underscore the importance of species discovery by emphasizing the value of an integrative taxonomic approach, the effect of species delineation on biodiversity, and the necessity of properly vouchered specimens.

While historical taxonomic work generally relied on morphological characters alone to delineate and typify species, modern taxonomy more often utilizes other biological evidence (Dayrat, 2005; Schlick-Steiner et al., 2010). An integrative approach is frequently used to overcome the challenges of cryptic species, especially those lacking clear morphological characters adequate for recognizing species boundaries. Complementary lines of evidence in addition to morphology (e.g., behavioral, molecular, chemical, ecological, etc.) increase our confidence in species descriptions and reveal the intricacies of those species' biology (Dayrat, 2005). Employing this approach, we analyzed biologically relevant evidence along with key morphological characters —summarized in the diagnoses of *M. mikromelanos* and *M. zeteki*—that proved useful for distinguishing the two species. These are best observed using a standard dissection microscope but can also be detected with a 20~~x~~X loupe. Another line of evidence is provided by our behavioral analysis. It was initially assumed that tempo would reflect behavioral differences observed in the field, where *M. zeteki* appeared 'aggressive' and *M. mikromelanos* 'passive'. However, we found that these two sibling species show differences in tempo, the rate of movement, rather than in aggressive or passive behaviors. Lastly, our chemical analysis also shows species-specific differences in the abundance of volatile compounds for workers. The combined evidence supports the existence of two distinct and closely related sympatric species in the Panama Canal Zone, *M. mikromelanos* and *M. zeteki*. The recognition of two species adds to our understanding of the multiple symbiotic relationships involving each species. It should be noted that, although it appears fairly certain that *M. mikromelanos* represents a single, well-supported species (Fig. 1e), the possibility remains that *M. zeteki* as currently defined may actually consist of two or more cryptic species. In Fig. 1e, all the samples of *M. mikromelanos* form a very well-supported clade whereas the monophyly of the two *M. zeteki* samples is poorly supported. This is also reflected in a larger phylogeny where the same two *M. zeteki* samples are

monophyletic but have similarly poor support and long branch lengths (see Fig. 2 of Solomon et al., 2019).

Species delimitation is essential not only for descriptive biology, but also for understanding the levels of biodiversity. In this context, species represent units of study that help us comprehend ecological and evolutionary principals. These include, but are not limited to, genetic diversity, adaptation, and broad-scale community interactions. Fungus-growing ants are an intriguing group for the study of biodiversity given their coevolutionary history with their fungal cultivars (Mehdiabadi et al., 2012), their many other symbiotic relationships (Mueller, Rehner & Schultz, 1998; Currie, Mueller & Malloch, 1999; De Fine Licht & Boomsma, 2014), and the role fungus-growing ants play as ecosystem engineers (Jones, Lawton & Shachak, 1994; Folgarait, 1998; Meyer et al., 2011, 2013). However, the distributions and ecological roles of most non-leaf-cutting attines in neotropical environments is still poorly studied (but see Leal & Oliveira, 2000; Vasconcelos et al., 2008; Tschinkel & Seal, 2016). For example, during the summer of 2018 we searched BCI, Fort Sherman, and El Llano (ca. 15, 35, and 80 km from Pipeline Road, respectively) for both *M. mikromelanos* and *M. zeteki*. Yet after searching kilometers of trails and creeks on BCI we were unable to locate any *M. mikromelanos* colonies, and only located two *M. zeteki* colonies on BCI and one at El Llano. No *M. mikromelanos* were found outside of the regularly sampled Gamboa Forest and Pipeline Road areas with the exception of a sample collected by Dr. Jack Longino in the Darien Provence of Panama in 2015. Regardless of our uncertainty of *M. mikromelanos*' distribution outside of the Canal Zone, we do have some familiarity with *M. mikromelanos*' and *M. zeteki*'s symbiotic associations. For example, they maintain similar relationships with social parasites, garden pathogens, and parasitoids (see "Biology" in species descriptions). Describing *M. mikromelanos* has enhanced our understanding of the symbiotic relationships of both species and raises more questions about them and their associates. Further research clarifying the natural history of these species and their symbionts will help us discern their ecological roles and contribute to our understanding of biodiversity in the Panama Canal Zone.

Genetic patterns and genetic diversity are another important aspect of biodiversity. Together they can inform understanding of the dispersal capabilities of species (Sanetra & Crozier, 2003; Sanllorente, Ruano & Tinaut, 2015; Boulay et al., 2017; Helms, 2018) biogeographic histories (Branstetter et al., 2017; Mueller et al., 2017, 2018), demographic history (Castilla et al., 2016), and evolutionary patterns (Baer & Boomsma, 2004; Schultz & Brady, 2008; Nygaard et al., 2016; Mueller et al., 2018). Modern molecular genetic tools enable researchers to study populations and their patterns at broad biogeographic ranges. For example, through biogeographic studies we know higher-attine ants grow two clades of higher-attine fungi, Clade A, the species *Leucoagaricus gongylophorus*, and Clade B, consisting of multiple unnamed species (Mueller et al., 2018). Yet, there is not a one-to-one association or phylogenetic congruence between higher-attine ants and their cultivars (Mueller et al., 2018). By including a broader distribution of both higher- and lower-attine species, it was found that some leaf-cutting species previously thought to grow only *L. gongylophorus* (Clade A) also grow Clade B

cultivars, previously thought to only be grown by non-leaf-cutting higher-attines (Mueller et al., 2018). Moreover, in both lower and higher attines, multiple species of cultivars can be cultivated by the same ant species in the same location and distantly related ant species, across broad geographic regions, can cultivate the same cultivar species (Green, Mueller & Adams, 2002; Mehdiabadi et al., 2012; Shik et al., 2020). As in most scientific endeavors, new knowledge of ant-fungus associations requires constant updating of older models (Chapela et al., 1994; Mueller & Wcislo, 1998; Schultz & Brady, 2008; Mehdiabadi & Schultz, 2010). This process generates a deeper and more complicated picture of the biogeographic patterns observed in populations of the higher attines. Well-designed population-level analyses of the 61 non-leaf-cutting higher-attine ant species (e.g., *Mycetomoellerius*, *Paratrachymyrmex*, *Trachymyrmex*, *Xerolitor*, and *Sericomyrmex*) would further refine our understanding of coevolution in the fungus-growing ants. *Mycetomoellerius mikromelanos* is well suited for such population-genetic analyses for a few reasons: it is abundant in the Canal Zone and easily located given its characteristic auricle nest entrance, it is sympatric with its sister species *M. zeteki*, and it has a published genome (Nygaard et al., 2016). Originally named *Trachymyrmex zeteki* on GenBank (Nygaard et al., 2016; GenBank accession: GCA_001594055.1), we confirm in this study based on published nuclear gene sequences (see Pphylogenetic Aanalysis) and morphological evidence of vouchers (see tTaxonomy; Figs. S9, & S10) that it is the genome of *M. mikromelanos*.

The published genome of *M. mikromelanos* highlights the importance of species identification and voucher specimen deposition. Physical vouchers provide reproducibility and confidence in published findings. Curating physical collections, naming species, and creating molecular databases still depend on non-molecular taxonomic work (Dayrat, 2005; Turney et al., 2015). We found that the incidence of reported vouchering for *M. zeteki* or *M.* cf. *zeteki*, based on our literature review, is higher than what is typically found in the field of entomology (44% versus 35%: Turney et al., 2015). This could be due to the exponential increase in research focusing on attines and collaborations with skilled taxonomists over the past thirty to forty years. We argue that more effort in voucher deposition is needed and that this is especially true when genomic information is published. Genomic resources are frequently used to compare and characterize gene functions (e.g., Lee et al., 2017; Nolasco et al., 2018; Wang et al., 2019). Incomplete taxonomic information can lead to a series of misguided future studies.

Conclusions

Given the abundance of *M. mikromelanos* in the Panama Canal Zone, we expect that the majority of researchers who believe they have studied *M. zeteki* have studied *M. mikromelanos* instead (Appendix Table 1). We encourage these researchers to mount specimens, confirm the species identification, and deposit the vouchers in a well-curated and accessible natural history museum collection. Our hope is that our results will encourage voucher deposition, even for common species such as *M. mikromelanos*. While physical voucher specimens are not typically required by journal policy or by reviewers (Turney et al., 2015), our findings draw attention to why this is important. We recommend that investigators include voucher specimen preparation and

deposition as part of their normal research practice and instill this principle in mentees and colleagues.

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