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An updated molecular phylogeny of the stingless bees of the genus *Trigona* (Hymenoptera, Meliponini) of the northern Peruvian forests

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Abstract

Stingless bees (Hymenoptera, Meliponini) are a large and diverse group including 59 extant groups, representing the main pollinators of Amazon forests. Among those, *Trigona* is one of the largest endemic genera of Neotropical Meliponini. In this work, we updated the molecular phylogeny of *Trigona* proposed by Rasmussen and Camargo (2008), including data from 59 new specimens collected in 2020 in the forests of northern Peru, through a multigene phylogenetic approach combining sequences from four gene fragments (16S, ArgK, EF-1a, opsin). Our results confirmed the monophyly of *Trigona* and of all proposed subgenera, except *Aphaneura*. In addition, most *Trigona* species-groups resulted monophyletic but the '*spinipes*' and '*pallens*' groups appeared paraphyletic and polyphyletic, respectively. Moreover, the cohesion of the "*fulviventris*" species group was hindered by the inclusion of *T. williana* (previously included in the "*pallens*" group) within this clade. Finally, we provided further evidence for a subdivision into two (geographically) distinct clades within *T. guianae* in northern Peruvian Amazon, which highlighted the importance of Neotropical biogeographical barriers in Meliponini divergence and evolution. Finally, to avoid misidentifications of *Trigona* specimens, the need for a robust taxonomic revision based on a cladistic approach of the whole genus is discussed.

Keywords

Apoidea, Neotropical biogeography, Peruvian Amazon, Taxonomy

Introduction

Stingless bees (Hymenoptera, Meliponini) are major pollinators in tropical forests (Roubik 1989) with about 623 species belonging to 59 extant and fossil groups (considered as genera, subgenera or synonymized depending on the classification) (Engel et al. 2023). Meliponini are distributed throughout the tropical and subtropical areas of the Afrotropical, Australasian, Indo-Malayan and Neotropical Regions, exhibiting the highest diversity in the New World Amazonian rainforests (Michener 1978; Roubik 1993; Michener 2007). Currently, 26 extant genera are considered endemic to the New World (Engel et al. 2023). Among these, *Trigona* Jurine, 1807 is exclusive to the Neotropics and is one of the largest genera of stingless bees (Michener 2007; Rasmussen and Cameron 2007). Recent molecular phylogenetic data confirmed the monophyly of the New World species of *Trigona*, a genus with 32 currently considered valid species (Camargo et al. 2013; Costa et al. 2003; Rasmussen and Cameron 2007; Rasmussen and Camargo 2008). Nine species-groups have been recognized based on morphological, ecological and distributional data, and largely supported by genetic analyses (Rasmussen and Camargo 2008). More recently, seven of these species-groups were elevated to subgenera of *Trigona* (Engel 2021) [i.e., '*cilipes*' as *Aphaneuropsis* Engel, 2021; '*fulviventris*' as *Koilotrigona* Engel, 2021; '*crassipes*' as *Necrotrigona* Engel, 2021; '*pallens*' as *Aphaneura* Gray, 1832; '*dimidiata*' as *Dichrotrigona* Engel, 2021; '*fuscipennis*' as *Ktinotrofia* Engel, 2021; '*recursa*' as *Nostotrigona* Engel, 2021], with the remaining two groups, '*amalthea*' and '*spinipes*' forming the subgenus *Trigona s. str.* Jurine, 1807.

This group of bees, characterized by small to large workers (5.5–11 mm), shows a variety of defense behaviors and nesting habits (i.e. nests are built on branches of plants or walls, in anthills or underground; Costa et al. 2004), as well as different foraging ecologies, from pollen and nectar gatherers (Fig. 1) to obligated necrophages (i.e., *Trigona crassipes* Fabricius, 1793, *T. hypogea* Silvestri, 1902 and *T. necrophaga* Camargo & Roubik, 1991; Roubik 1982; Camargo and Roubik 1991).

About 22 species of *Trigona* have been reported in Peru (Rasmussen and Gonzalez 2009; Camargo et al. 2013; Sánchez Sandoval et al. 2015; Castillo-Carrillo et al. 2016; Rasmussen and Delgado 2020), but the overall number is likely underestimated because many forested areas of the country remain unexplored.

Recently, several *Trigona* specimens dwelling in humid and seasonally dry forests of northern Peru (in San Martin and Piura regions) were identified through an integrative taxonomy approach, i.e., considering both morphology and COI barcoding (Marconi et al. 2022). As expected, the COI-based reconstructed phylogeny was mostly unresolved at deep nodes. In addition, the newly collected Peruvian specimens ascribed to *T. fulviventris* Guerin-Meneville, 1845 and *T. guianae* Cockerell, 1912 were split into four distinct clades, two for each species (named provisionally as 'A' and 'B' clades in both cases). The same phylogenetic analysis also detected two lineages that were unrelated to other identified species, which were provisionally attributed to *T.* sp. 1 and *T.* sp. 2, respectively (Marconi et al. 2022).

Figure 1. *Trigona* cf. *chanchamayoensis* Schwarz, 1948 sucking nectar from a flower (Photo M. Marconi).

In this work we conducted a multigene phylogenetic analysis of the Neotropical genus *Trigona* by integrating novel molecular data of four genes obtained from northern Peruvian specimens (Marconi et al. 2022) with a previously published dataset (Rasmussen and Camargo 2008). By updating the current phylogeny, we aimed to clarify the taxonomic issues emerged in our previous work (Marconi et al. 2022) and further validate the currently recognized species-groups within *Trigona* (Rasmussen and Camargo 2008) and the recently proposed subgenera (Engel 2021).

Methods

59 specimens of *Trigona* were collected in 2020 in five Northern Peruvian forests, all located east of Andes except Mangamanguilla [Juliampampa (JP) (800–110 m a.s.l. and -6°26'3.5556"N, -76°19'47.5896"E), Pabloyacu (PY) (950–1200 m a.s.l. and -6°4'6.3984"N, -76°56'24.8388"E), Pongo de Cainarachy (POA) (150–550 m a.s.l. and -6°21'22.608"N, -76°17'3.174"E), Utcurarca (UT) (250–550 m a.s.l. and -6°39'43.7616"N, -76°17'0.438"E) and Mangamanguilla (MA) (140–450 m a.s.l. and -5°18'46.5228"N, -79°51'51.084"E)] and tentatively assigned through an integrative taxonomic approach (i.e. combining morphology and COI barcoding, after a 'salting-out' DNA extraction from one middle leg) to ten different species (Marconi et al. 2022). PCR was conducted to amplify gene fragments of mitochondrial 16S rRNA (16S), nuclear long-wavelength rhodopsin copy 1 (opsin), elongation factor-1a copy F2 (EF-1a), and arginine kinase (ArgK) using published primers (Rasmussen and Cameron 2007; Rasmussen and Camargo 2008). The total reaction volume (25 µl) contained 0.5 pmol of each primer, 10 mM Tris-Cl, pH 8.3 and 50 mM KCl, 1.5 mM $MgCl_2$, 2.5 mM dNTPs, 2 µl of the DNA template and 1 unit of Taq DNA polymerase (Meridian). PCR cycling conditions consisted of an initial denaturation of

3 min. at 94 °C followed by 35 cycles of 30 sec. at 94 °C, 30 sec. at 50 °C and 1 min. at 72 °C, and a final elongation step of 10 min. at 72 °C. Products were visualized on a 1% agarose gel stained using Midori Green Advance dye (Nippongenetics). PCR products were purified using the ExoSAP-IT PCR Product Cleanup Reagent (Applied Biosystem) and sent to the sequencing facility of Microsynth AG (Switzerland).

DNA sequences were edited and aligned with STADEN PACKAGE 2.0.0b11-2016 (http://staden.sourceforge.net/). Sequences (including those of outgroup taxa) from Rasmussen and Camargo (2008) were downloaded and aligned with our data using MAFFT v1.4.0 (Katoh and Standley 2013) to produce comprehensive datasets. Phylogenetic analyses were conducted with Maximum Likelihood (ML) and Bayesian Inference (BI) on both single gene and combined datasets. For both ML and BI approaches, ModelFinder (Kalyaanamoorthy et al. 2017) implemented in IQ-TREE v 1.6.12 (Nguyen et al. 2015) was used to find the best substitution model for each gene (= partition) according to the BIC criterion. ML analyses were performed with IQ-TREE v 1.6.12 (Nguyen et al. 2015) setting 2000 replicates to estimate node supports with ultrafast bootstrap (UFBboot2; Hoang et al. 2018). MRBAYES v3.2.7a (Ronquist et al. 2012) was used for Bayesian Inferences by running two MCMC and four chains for 10 million generations with a default (25%) burn-in. Trees were sampled every 1000 generations, and convergence assessed with Tracer v1.6 (Rambaut et al. 2014). FIGTREE v1.3.1 (Rambaut and Drummond 2009) was used to inspect the obtained trees. Only clades with UFBoot (UFB) values ≥ 95% (Minh et al. 2013) and posterior probability (PP) values ≥ 0.95 (Erixon et al. 2003) were considered as strongly supported upon analyses. All voucher specimens were deposited in Estudios Amazonicos Biological Material Depositary Center (Tarapoto, Peru) (Marconi et al. 2022).

Results

We obtained 58 sequences of 16S (Genbank Acc. n° OR353456–OR353513), 26 of ArgK, 41 of EF-1a and 26 of opsin (Genbank Acc. n° OR393480–OR393571) from a total of 59 northern Peruvian *Trigona* specimens collected in 2020 (Marconi et al. 2022). The combined dataset, including previously generated sequences of *Trigona* and outgroup species (Rasmussen and Camargo 2008), consisted of a total of 88 individuals (including 5 outgroups) with 2329 aligned positions composed by the four gene fragments: 485 base pairs (bp) of 16S, 592 bp of ArgK, 729 bp of EF-1a and 522 bp of opsin gene. ML and BY tree topologies largely overlapped (hence, BY topology only is shown; Fig. 2). The combined ML and BY analysis confirmed the monophyly of the genus *Trigona* (Fig. 2: PP = 1.00/UFB = 100) and the presence of two main distinct clades, one (PP = 1.00/UFB = 98) including members of the '*amalthea*' + '*spinipes*' (= *Trigona s. str.*), '*fuscipennis*', (= *Ktinotrofia*) '*recursa*' (= *Nostotrigona*) and '*crassipes*' (= *Necrotrigona*) species groups (or subgenera) (PP = 1.00/UFB = 100), the other including members of the '*cilipes*' (= *Aphaneuropsis*), '*pallens*' (= *Aphaneura*) and '*fulviventris*' (= *Koilotrigona*) species groups (or subgenera) (Rasmussen and Camargo 2008; Engel

2021) (Fig. 2). Five out of the 8 *Trigona* species groups were recovered as monophyletic: '*amalthea*' (PP = 1.00/UFB = 99), '*fuscipennis*' (PP = 1.00/UFB = 100), '*recursa*' (also including *T.* sp. 1; PP = 1.00/UFB = 98), '*crassipes*' (PP = 1.00/UFB = 98), '*cilipes*' (PP = 1.00/UFB = 100). The '*spinipes*' group appeared paraphyletic since it was split into two distinct, though poorly supported clades (Fig. 2). One included *T. spinipes*, *T. hyalinata*, *T. corvina* and *T. amazonensis* ('*spinipes*' *1*; PP = 0.80/UFB = 94), whereas the other grouped *T. nigerrima* and *T. dallatorreana* (= *T.* sp. 2) ('*spinipes*' *2*; PP = 0.93/UFB = 71). However, all '*spinipes*' members clustered with those of '*amalthea*', thus supporting the monophyly (PP = 1.00/UFB = 100) of the subgenus *T.* (*Trigona s. str.*) *sensu* Engel 2021. *T. williana* did not cluster within the '*pallens*' group, but was genetically closer to members of the '*fulviventris*' group (= *Koilotrigona*). However, its placement within the '*fulviventris*' group or *Koilotrigona* subgenus remains doubtful since it received a low Bayesian support (PP = 0.52; Fig. 2). Hence, the '*pallens*' group and the subgenus *Aphaneura* Gray 1832 (Engel 2021) are tenable only if *T. williana* is excluded and placed in a different group/subgenus, still to be defined. Finally, as already reported (Marconi et al. 2022), Peruvian specimens of *T. guianae* were subdivided into two well-supported and distinct clades (A and B) (Fig. 2), whereas those ascribed to *T. fulviventris* were included in the same clade (A+B; see Marconi et al. 2022).

Discussion

We here built upon the molecular phylogeny of the Neotropical genus *Trigona* (Rasmussen and Camargo 2008) by adding genetic data from newly collected specimens in northern Peruvian forests (Marconi et al. 2022).

We confirmed the monophyly of the Neotropical genus *Trigona* and of all proposed subgenera, except for *Aphaneura* Gray, 1832 (Engel 2021). In addition, most *Trigona* species-groups were found to be monophyletic (Fig. 2). However, as already observed (Rasmussen and Camargo 2008), the '*spinipes*' and '*pallens*' species groups were paraphyletic and polyphyletic, respectively (Fig. 2). Our results support combining members of the '*amalthea*' and '*spinipes*' groups into the proposed subgenus *T.* (*Trigona s. str.*). However, the closely related *T.* (*Trigona*) *dallatorreana* (= *T.* sp. 2; Marconi et al. 2022) and *T.* (*Trigona*) *nigerrima* should be ascribed to a different species-group (provisionally named '*spinipes*' 2 in Fig. 2). As previously mentioned, the '*pallens*' group (= *Aphaneura*) as usually recognized is polyphyletic due to the large genetic distance of *T. williana* from all other members of this group/subgenus. In fact, *T. williana* is similar only in coloration to members of the '*pallens*' group and differs in the shape of metasoma and metatibiae (F.F. De Oliveira, pers. comm.). The placement of *T. williana* within the '*fulviventris*' group is also doubtful as it differs in many morphological and biological features from other members of the group. Its true placement will require further investigation. In general, since some taxonomic issues affect the '*pallens*' group (e.g., the types of both *T. muzoensis* Schwarz, 1948 and *T. ferricauda* Cockerell, 1917 should be re-examined to exclude possible synonymies), we cannot rule out that

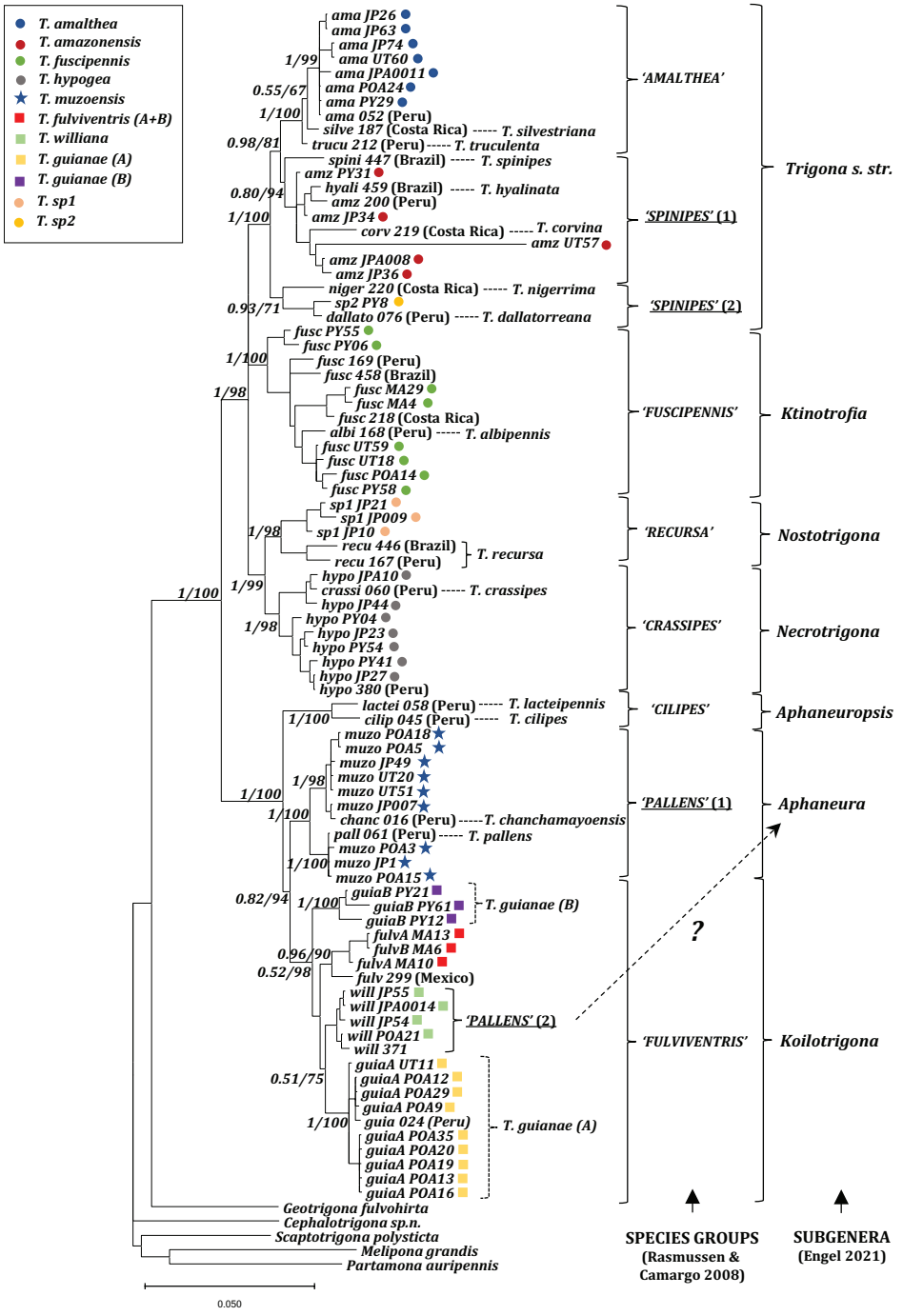

Figure 2. *Trigona* Bayesian phylogenetic tree topology estimated from combined sequence data from four gene fragments (16S, ArgK, EF-1a, opsin). Posterior probability and ultra-fast bootstrap values (BY - PP/ML - UFB) are shown at deepest nodes only. Color marks are assigned to tips leading to the 59 northern Peruvian specimens belonging to *Trigona* species, whose taxonomic identification and geographic origin are reported in detail in table 1 of Marconi et al. 2022.

our specimens, formerly recognized through the integrative taxonomic approach as *T. muzoensis* (Marconi et al. 2022), could be instead ascribed to *T. chanchamayoensis* Schwarz, 1948 - occurring in Peru east of Andes (type locality: San Ramon, Valle de Chanchamayo, Peru) - or to *T. pallens* Fabricius, 1798. In fact, the specimens morphologically identified by Rasmussen and Camargo (2008) as *T. chanchamayoensis* and *T. pallens* (i.e., chanc 016 and pall 061; Fig. 2) are placed in two distinct clades including two separate groups of individuals previously identified as *T. muzoensis* (Marconi et al. 2022), respectively (Fig. 2). When these northern Peruvian specimens were identified in BoldSystems (www.boldsystems.org) (Marconi et al. 2022), they received ID scores ranging 96.43 (POA) - 98.46% (e.g., JP007) for *T. muzoensis*, but did not match with the single *T. chanchamayoensis* available in BoldSystems (from Brazil), nor with *T. pallens*, totally lacking COI data. Unfortunately, taxonomic keys to promptly distinguish morphologically all members of the '*pallens*' group are also lacking. Similarly, doubts could be raised to our previous attribution of northern Peruvian specimens to *T.* cf. *hypogea* or *T.* cf. *fuscipennis* (Marconi et al. 2022), because these show a close (although scarcely supported) phylogenetic relatedness to two species identified by Rasmussen and Camargo (2008), i.e., *T. crassipes* (crassi 060) and *T. albipennis* (albi 168), respectively (Fig. 2). However, for these two species as well, data are lacking in BoldSystems, nor valuable keys of distinctive morphological characters are available for '*crassipes*' and '*fuscipennis*' groups. In general, the absence of published dichotomous keys based on reliable diagnostic morphological characters and cladistic approaches integrating extensive genetic (COI or other marker) datasets aimed to define species boundaries, still hinder the correct identification of stingless bee species (see also, Marconi et al. 2022). These data deficiencies are likely to generate conflicts in *Trigona* identification (as, in this case, with those of Rasmussen and Camargo 2008) and favor the description of new species without truthfully considering their morphological and genetic internal cohesion, as well as their distinction from other (sibling) taxa.

We also confirmed a genetic subdivision within *T. guianae* into two putatively distinct taxonomic and/or geographic units, possibly originated by limited gene flow due to biogeographic barriers in the Neotropics (Marconi et al. 2022). Indeed, comparative analysis of metatarsi of *T. guianae* (Clade A) and *T. guianae* (Clade B) revealed morphological differences at the retrodorsal margin and distal angle (unpublished data). Further data will allow establishing if *T. guianae* (Clade B) could be ascribed to a novel species endemic to Pabloyacu, or to one of the approximately 28 novel species awaiting description (Rasmussen and Camargo 2008). On the other hand, the combined molecular dataset did not support the split into two distinct entities in *T. fulviventris*, as previously suggested based on COI marker only (Marconi et al. 2022). However, a recent morphological analysis showed that *T.* cf. *fulviventris* (Clade A) has a narrow subtriangular metatibia, whereas *T.* cf. *fulviventris* (Clade B) (MA6) has a broad, "drop-like" shape (unpublished data). Additional specimens will be examined, both genetically and morphologically, to clarify such issues.

Concerning the two previously unidentified *Trigona* species (Marconi et al. 2022), as reported above we confirm that *T.* sp. 2 is *T. dallatorreana*, whereas *T.* sp. 1 seems to be related to *T. recursa*, although its taxonomic relationships need further examination.

Novel genetic/genomic data from populations sampled across the entire geographic ranges of all of the *Trigona* species groups will shed light on the phylogenetic relationships among members of this large genus of Neotropical stingless bees. Further morphological work is also needed to produce and/or refine taxonomic keys and accurately revise the taxonomy of this speciose genus. Such an effort would not only resolve some taxonomic issues within this large genus of stingless bees, but also enhance our understanding of the role of Neotropical biogeographic barriers in the evolution of this main group of pollinators of the Amazon forests.

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