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A molecular phylogeny of the stingless bee genus *Melipona* (Hymenoptera: Apidae)

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ABSTRACT

Stingless bees (Meliponini) constitute a diverse group of highly eusocial insects that occur throughout tropical regions around the world. The meliponine genus *Melipona* is restricted to the New World tropics and has over 50 described species. *Melipona*, like *Apis*, possesses the remarkable ability to use representational communication to indicate the location of foraging patches. Although *Melipona* has been the subject of numerous behavioral, ecological, and genetic studies, the evolutionary history of this genus remains largely unexplored. Here, we implement a multigene phylogenetic approach based on nuclear, mitochondrial, and ribosomal loci, coupled with molecular clock methods, to elucidate the phylogenetic relationships and antiquity of subgenera and species of *Melipona*. Our phylogenetic analysis resolves the relationship among subgenera and tends to agree with morphology-based classification hypotheses. Our molecular clock analysis indicates that the genus *Melipona* shared a most recent common ancestor at least ~14–17 million years (My) ago. These results provide the groundwork for future comparative analyses aimed at understanding the evolution of complex communication mechanisms in eusocial Apidae.

1. Introduction

The stingless bee genus *Melipona* contains at least 50 species of medium-sized (8–15 mm), robust, and often hirsute bees inhabiting forests of tropical America, from Mexico to Argentina (Schwarz, 1932; Michener, 2007). Most species of *Melipona* inhabit lowland wet forests, with the greatest species diversity concentrated in the Amazon Basin (Moure and Kerr, 1950). These bees are highly eusocial, which means they exhibit reproductive division of labor, cooperative brood care, and overlap of generations (Wilson, 1971).

Similar to honey bees (*Apis*), *Melipona* are remarkable for insects, in their ability to recruit nest mates to specific foraging sites (von Frisch, 1967; Michener, 1974; Dyer, 2002; Nieh, 2004). All *Apis* use a form of referential communication known as the waggle dance, whereby returning foragers inform colony members about newly discovered resource sites (von Frisch, 1967; Seeley, 1995; Dyer, 2002). The waggle dance communicates distance and direction (von Frisch, 1967; Gould, 1976; Michelsen et al., 1992; Esch et al., 2001; Dyer, 2002; Sherman and Visscher, 2002). The communication mechanisms of *Melipona* are less studied, but experimental evidence indicates functional referential communication in some species (Esch, 1967; Aguilar and Briceño, 2002; Nieh, 2004), but not in others (Hrncir et al., 2006). Upon returning to the nest, successful *M. panamica* and *M. seminigra* foragers may perform short piloting flights outside of the nest in the direction of the resource (Nieh, 1998; Nieh and Roubik, 1998), while inside the nest, they produce sound pulses while distributing food samples to potential recruits (Esch, 1967; Nieh, 2004). The average duration of sound pulses correlates with, and thus potentially encodes, distance to food sources relative to the location of the nest (Esch, 1967; Nieh and Roubik, 1998). Additionally, there are differences in the ability to communicate different spatial dimensions among species of *Melipona*, which correlate well with spatial distribution of floral resources in their current environment (Nieh et al., 2003). Whether and how this information is actually utilized by nest mates is still a subject of intense investigation, as was the case for decades in *Apis*.

Although the genus has been the focus of behavioral, genetic, ecological, and pollination studies (Roubik, 2006), only partial phylogenetic analyses have been carried out to date (Rego, 1990; Costa et al., 2003; Fernandes-Salomão et al., 2005; Rasmussen and Cameron, 2010). The stingless bee genus *Melipona* is clustered within

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the Neotropical Meliponini (Rasmussen and Cameron, 2010), and its monophyly is well supported (Rego, 1990; Costa et al., 2003; Fernandes-Salomão et al., 2005). A recent global phylogenetic analysis of the entire tribe Meliponini supported a Miocene (~24 My) origin for *Melipona*, but only 20 of the 50 described species were sampled and the internal relationships were not well resolved (Rasmussen and Cameron, 2010). Here, we present the first comprehensive species-level phylogenetic analysis of *Melipona* coupled with a molecular clock analysis.

2. Materials and methods

2.1. DNA sequencing and taxonomic sampling

We sequenced ~4.5 kb of DNA from five different fragments including mitochondrial *CO1* (~1.2 kb), ribosomal *16S* (~0.6 kb), nuclear *EF1-α* (~1.2 kb), *ArgK* (~0.7 kb), and *Pol-II* (0.8 kb). DNA was extracted from individual bee specimens from either leg or thoracic muscle tissue using Qiagen DNA Extraction Kits (Qiagen Inc., Valencia, California). Polymerase Chain Reactions (PCRs) were carried on a Bio-Rad DNA Engine Dyad® Peltier thermal cycler (Bio-Rad Laboratories Inc., Hercules, California) in 25 μL reactions with 2.5 mmol/L $MgCl_2$, 2.5 mmol/L PCR buffer, and *Taq* polymerase (Qiagen Inc., Valencia, California) using various primer pairs (Danforth et al., 2004; Supplementary Table 1). We purified PCR products by incubating samples at 37 °C for 35 min using *Escherichia coli* Exonuclease I enzyme (New England Biolabs, Hanover, Maryland) and subsequently raising the temperature to 80 °C for 20 min. Purified products were cycle-sequenced using BigDye™ Terminator v3.1 Ready Reaction Cycle Sequencing Kit (Applied Biosystems Inc., Foster City, California). Samples were directly sequenced on an Applied Biosystems Inc., 3100 Genetic Analyzer (Applied Biosystems Inc., Foster City, CA). Both forward and reverse strands were sequenced for each of the five markers; complementary strands were assembled using the software Sequencher™ v4.2 (Gene Codes Corp., Ann Arbor, MI).

All major lineages within *Melipona* and *Apis* were sampled for this study, including four subgenera, 35 species, and 51 individuals of approximately 50 described species of *Melipona* representing all main species groups, and three subgenera, six species, and 10 individuals of *Apis*. Additionally, we sampled 30 taxa within the corbiculate bees, including bumble bees, stingless bees, and orchid bees, and two outgroups (*Epicharis* and *Centris*). We include a total of 88 terminals. GenBank accession numbers are provided in Supplementary Table 3.

2.2. Phylogenetic analyses

A single DNA matrix containing five loci was assembled using MacClade v4.06 (Maddison and Maddison, 2003). Parsimony analyses were implemented in the software package Paup* v4.0b (Swofford, 2003) with all characters weighted equally and transitions assumed unordered. We performed 100 random addition sequences using the TBR algorithm, and estimated node support via non-parametric bootstrapping (100 replicates). A Maximum Likelihood (ML) phylogenetic analysis was performed in the software package GARLI (Zwickl, 2006) with model parameters estimated over the specified number of runs. Bootstrap support values were estimated in GARLI with 100 heuristic tree searches using the same parameters as those implemented during tree searches. Additionally, Bayesian analyses were implemented in the software package MrBayes v3.1.1 (Ronquist and Huelsenbeck, 2003). Bayesian tree searches were made assuming both single (GTR+Γ+I) and multiple models of sequence evolution for each locus (see Supplementary Table 2). In addition, we ran a tree search where models of sequence evolution were partitioned by codon positions, with parameters estimated separately for first, second, and third codon positions of nuclear coding genes. Markov chain Monte Carlo (MCMC) searches were run for 10,000,000 generations, sampling every 1000 generations for a total of 10,000 trees; model parameters were estimated during the run. Three parallel runs were carried, and for each run one unheated and three incrementally heated chains were used. We checked for convergence within tree searches by plotting tree likelihood values against the number of generations, and among searches by comparing resulting topologies. Bayesian posterior probabilities were estimated as the proportion of trees containing each node over the trees sampled during runs. The trees corresponding to the first 1000 generations were discarded ("burn-in").

2.3. Divergence time estimation

Divergence times were estimated using a fully resolved topology obtained by applying a 50% Majority-Rule (MR) consensus to all the trees obtained from a Bayesian phylogenetic search; the remaining polytomies (six) were resolved randomly using the R software package APE v2.3. Using a Likelihood Ratio Test (LRT) we estimated this tree had a significantly lower score value (−nL 39222.08) when a molecular clock was enforced than when the assumption was relaxed (−nL 38987.29). We calculated branch lengths on the 50% MR consensus tree via maximum likelihood in the software package Paup*, optimized under the model of sequence evolution GTR+Γ+I (molecular clock not enforced). Node divergence times were estimated with Penalized Likelihood (PL) using the Truncated-Newton algorithm in the software package r8s v1.71 (Sanderson, 1997). Mean ages ± SD were calculated using non-parametric bootstrapping.

We used two sets of calibration ages, corresponding to the youngest and oldest estimates of the ages of the fossils used as node age constraints. A total of five different ages were used to calibrate our molecular clock trees (indicated by letters in Fig. 1): A, maximum root age (80–100 My, based on oldest stem bee fossil (Poinar and Danforth, 2006) and molecular clock analysis done by Hines (2008)); B, *Cretotrigona prisca* (65–70 My, Michener and Grimaldi, 1988; Engel, 2000) used as a minimum age calibration; C, *Euglossa moronei* (15–20 My, Engel, 1999b) used as a minimum age calibration; D *Apis lithohermaea* (14–16 My, Engel, 2006) used as a minimum age calibration; and E, *Proplebeia dominicana* (15–20 My, Wille and Chandler, 1964; Camargo et al., 2000) used as a minimum age calibration. Although the age of *C. prisca* has been the subject of controversy (Michener and Grimaldi, 1988; Engel, 2000), this fossil exhibits synapomorphic characters that unambiguously place it within crown Meliponini. Thus, we used its age as a minimum age calibration for all Meliponini. The placement of *E. moronei* within extant (crown) *Euglossa* is justified by the presence of elongated mouthparts, labrum shape, and pubescence (Engel, 1999b). The phylogenetic position of *A. lithohermaea* within extant *Apis* is justified by the enlarged body size, elongated metabasitarsus, wing venation, and infuscated wing membrane (Engel, 2006). The placement of *P. dominicana* within extant Neotropical Meliponini is justified by the short trapezoidal clypeus, triangular shape of forewing medial cell, and shape of tibiae and basitarsi (Camargo et al., 2000). The concordance among calibration points was assessed with the cross-validation method (Near et al., 2005; Supplementary Fig. 3). Since our phylogenetic sampling included divergent extant lineages within *Apis* (Raffiudin and Crozier, 2007), *Euglossa* (Ramírez et al., in press) and Neotropical Meliponini (Rasmussen and Cameron, 2010) we used fossil ages as minimum age constraints, even though in some cases lineage sampling was incomplete (e.g. *Euglossa*).