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Original article

A molecular phylogeny and the evolution of nest architecture and behavior in *Trigona s.s.* (Hymenoptera: Apidae: Meliponini)*

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Abstract – Stingless bees exhibit extraordinary variation in nest architecture within and among species. To test for phylogenetic association of behavioral traits for species of the Neotropical stingless bee genus *Trigona s.s.*, a phylogenetic hypothesis was generated by combining sequence data of 24 taxa from one mitochondrial (16S rRNA) and four nuclear gene fragments (long-wavelength rhodopsin copy 1 (opsin), elongation factor-1α copy F2, arginine kinase, and 28S rRNA). Fifteen characteristics of the nest architecture were coded and tested for phylogenetic association. Several characters have significant phylogenetic signal, including type of nesting substrate, nest construction material, and hemipterophily, the tending of hemipteroid insects in exchange for sugar excretions. Phylogenetic independent habits encountered in *Trigona s.s.* include coprophily and necrophagy.

molecular phylogeny / neotropical / stingless bee / behavior / nest

1. INTRODUCTION

Among the social bees there exists a bewildering array of nest architecture and nesting behavior, from the simple underground tunnels of halictid bees to the elaborate structures and forms of stingless bees (Michener, 2000). Nest characteristics such as the nesting site, architectural complexity, and building materials may be taxon specific and provide an excellent opportunity to assess information about underlying behavioral evolution of the respective taxa and higher groups (Michener, 1961, 1964; Kerr et al., 1967; Wille and Michener, 1973; Wenzel, 1991). Within the stingless bees, the largest and most diverse group of social bees worldwide, the external nest entrance and internal nest features often exhibit details that allow for species-specific recognition (e.g., Michener, 1959; Sakagami, 1982; Camargo and Pedro, 2003b; Franck et al., 2004). Recent reviews of the diverse biological features of stingless bees include their multi-faceted recruitment communication (Nieh, 2004) and the architectural diversity and complexity of their nests (Roubik, 2006), while new molecular phylogenies have elucidated generic relationships (Costa et al., 2003; Rasmussen and Cameron, 2007; Rasmussen, unpubl. data). These investigations provide a new foundation for the study of behavioral evolution in a comparative framework.

Previous comparative studies of stingless bee nesting biology (summarized in Wille and Michener, 1973; Roubik, 2006) provide

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important descriptive accounts. Phylogenetic hypothesis-based studies combining character data from morphology and nest architecture have been proposed for two Neotropical genera *Partamona* and *Trichotrigona* (Camargo and Pedro, 2003a, b). However, the use of nest characters in the reconstruction of phylogenies of stingless bees is difficult because of apparent homoplasy, with frequent reversals and independent gains of traits, such as the construction of combs in clusters (Wille and Michener, 1973). Rasmussen (2004) and Roubik (2006) further noted intraspecific plasticity in the choice of the nest substrate (i.e., physical placement of the nest), suggesting that species may behave opportunistically and employ a range of nest designs.

In general terms, nests of stingless bees are established in cavities where the bees may build solid "batumen plates" to shield and protect the colony. Nests are then constructed using wax in a mixture with resins, mud, feces, or other materials collected by the bees. The nest entrance provides access into the nest where the brood is located. "Involucrum sheaths" made of cerumen (a mixture of wax with resins) may be built as a protective layer or sheath around the brood chamber ("brood involucrum") or around the whole colony ("external involucrum"), including the storage vessels for honey and pollen. Brood cells can be clustered or they may be arranged in combs that are usually positioned in a horizontal plane. Outside the brood involucrum, small pots with food provisions are built in clusters. All of these nest characteristics (substrate, composition, and texture of the different parts of the nest), are variable across the stingless bees. Detailed descriptions of stingless bee nests with introductions to the terminology and diversity are available in several reviews (Schwarz, 1948; Michener, 1961; Wille and Michener, 1973; Sakagami, 1982; Wille, 1983; Camargo and Pedro, 2003b; Roubik, 2006).

The stingless bee genus *Trigona s.l.* has traditionally included subgenera from both the Old and the New World (Michener, 2000). Recently, Costa et al. (2003) and Rasmussen and Cameron (2007) circumscribed *Trigona* as a strictly New World genus, although its phylogenetic position among the remaining New World genera was not examined. *Trigona s.s.* occurs from Mexico (Nayarit and Sinaloa; *Trigona fulviventris*) to Argentina (Misiones; *Trigona spinipes*) and encompasses 32 nominal species and approximately 28 undescribed species (Camargo, unpubl. data). Most of the species were treated in the seminal taxonomic (and biological) revision of Schwarz (1948), with recent nomenclatorial changes summarized by Camargo and Pedro (2007). The species of *Trigona s.s.* can be placed into nine species groups (Tab. I) as recognized by Camargo (unpubl. data), based on their morphological and biological characteristics.

Our goals in this paper are to describe the likely evolutionary history and potential phylogenetic association of some of the diverse nest characteristics of an array of species of *Trigona s.s.* We describe and code 15 nest characters, and map these onto a new molecular phylogeny reported here. We interpret those characters that are phylogenetically associated as evolutionarily conserved and those that lack such association as evolutionarily labile.

2. MATERIALS AND METHODS

2.1. Taxon sampling and DNA sequencing

Twenty-four taxa were included from all but the *Trigona dimidiata* species group (Tab. II). Outgroups were represented by five New World taxa from Rasmussen and Cameron (2007). Voucher specimens used in the molecular analysis are deposited in the Illinois Natural History Survey (Champaign, USA). We did not sample exhaustively to include additional undescribed species within each species group (Tab. I), although *T. recursa* and *T. fuscipennis* were represented by multiple specimens in our analysis, as morphology suggests these represent distinct biological species. DNA sequences from gene fragments used in Rasmussen and Cameron (2007) (mitochondrial 16S rRNA, nuclear long-wavelength rhodopsin copy 1 (opsin), elongation factor-1α copy F2 (EF-1α), and arginine kinase (ArgK)) were obtained for inferring interspecific and intergeneric relationships. To strengthen support for relationships among the outgroups and their relationship to the

Table I. Nine recognized species groups of *Trigona s.s.* based on morphological and biological characteristics and their distribution. The distribution patterns are indicated according to the biogeographical areas or main Neotropical components that they occupy, as proposed by Camargo and Pedro (2003a: 371, Fig. 56a): "Atl" (Southeastern Brazil), "SEAm" (south of the rivers Madeira and Amazon to the northwest of Argentina and north of São Paulo, Brazil), "NAm" (craton of the northern Brazil, Guianas, Venezuela, and west of Colombia), "SWAm" (southwestern Amazon region, limited to the north by the rivers Negro and Uaupés, to the southeast, and east by the rivers Madeira/Mamoré, and to the west by the Andean mountain range), "Chocó-CA" (southwestern Ecuador north to the lowlands of Mexico). The distribution patterns correspond to: (A) wide distribution in Neotropical region: Atl, SEAm, NAm, SWAm, Chocó-CA; (B) restricted to the components NAm, SWAm, SEAm, with one or two species in central part of Chocó-CA (north of Colombia, Panama, and Costa Rica) and none in Atl; (C) restricted to the components NAm, SWAm, and northern part of SEAm.

Species group	Included nominal and undescribed taxa of *s.s.* in each species group	Distribution pattern *Trigona*
"*amalthea*"	*T. amalthea* (Olivier, 1789); *T. silvestriana* (Vachal, 1908); *T. truculenta* Almeida, 1984	A
"*fulviventris*"	*T. fulviventris* Guérin, 1835; *T. braueri* Friese, 1900; *T. guianae* Cockerell, 1910; ca. 6 undescribed species	A
"*fuscipennis*"	*T. fuscipennis* Friese, 1900; *T. albipennis* Almeida, 1995; ca. 8 undescribed species	A
"*spinipes*"	*T. spinipes* (Fabricius, 1793); *T. hyalinata* (Lepeletier, 1836); *T. nigerrima* Cresson, 1878; *T. dallatorreana* Friese, 1900; *T. pampana* Strand, 1910; *T. branneri* Cockerell, 1912; *T. corvina* Cockerell, 1913; *T. amazonensis* (Ducke, 1916); at least 3 undescribed species	A
"*cilipes*"	*T. cilipes* (Fabricius, 1804) (= *T. mazucatoi* Almeida, 1995); *T. lacteipennis* Friese, 1900; *T. pellucida* Cockerell, 1912; one undescribed species	B
"*crassipes*"	*T. crassipes* (Fabricius, 1793); *T. hypogea* Silvestri, 1902; *T. necrophaga* Camargo and Roubik, 1991; ca. 5 undescribed species	B
"*pallens*"	*T. pallens* (Fabricius, 1798); *T. williana* Friese, 1900; *T. ferricauda* Cockerell, 1917; *T. muzoensis* Schwarz, 1948; *T. chanchamayoensis* Schwarz, 1948; at least 2 undescribed species	B
"*dimidiata*"	*T. dimidiata* Smith, 1854; *T. venezuelana* Schwarz, 1948; *T. sesquipedalis* Almeida, 1984	C
"*recursa*"	*T. recursa* Smith, 1863; *T. permodica* Almeida, 1995; at least 3 undescribed species	C

ingroup, we also included sequences from the more conserved nuclear 28S rRNA (D2-D3 expansion regions and related core elements). DNA extraction, PCR, and sequencing protocols are reported in Rasmussen and Cameron (2007). The PCR amplification of 28S were performed using published primers (For28SVesp, Rev28SVesp: Hines et al., 2007) at an annealing temperature of 50 °C and extension at 72 °C. Both strands were sequenced for all taxa and consensus sequences were deposited in GenBank (accession numbers in Tab. II).

2.2. Phylogenetic methods

DNA sequences were edited and aligned in BioEdit version 7.0.0 (Hall, 1999). Default BioEdit alignments were adjusted by hand to optimize positional homology, in particular within introns and variable regions. Relationships were inferred from Bayesian analyses as implemented in MrBayes version 3.1.2 (Ronquist and Huelsenbeck, 2003). Genes were analyzed individually and collectively, with protein-coding genes partitioned

Table II. List of taxa included in the molecular phylogeny, including voucher code, collecting localities, and GenBank accession numbers.

Species	Voucher code	Collection locality	GenBank				
			16S	ArgK	EF-1a	Opsin	28S
Trigona albipennis	168	Peru, Junín	EU049705	EU049755	EU049778	EU049800	EU049728
Trigona amalthea	052	Peru, San Martín	DQ790468	DQ813070	EU049772	DX813227	EU049722
Trigona amazonensis	200	Peru, Madre de Díos	DQ790469	DQ813071	EU049781	DQ813228	EU049731
Trigona chanchamayoensis	016	Peru, San Martín	EU049698	EU049748	EU049769	EU049793	EU049719
Trigona cilipes	045	Peru, San Martín	EU049699	EU049749	EU049771	EU049794	EU049721
Trigona corvina	219	Costa Rica, Puntarenas	EU049710	EU049760	EU049784	EU049805	EU049734
Trigona crassipes	060	Peru, San Martín	EU049701	EU049751	EU049774	EU049796	EU049724
Trigona dallatorreana	076	Peru, San Martín	EU049703	EU049753	EU049776	EU049798	EU049726
Trigona fulviventris	299	Mexico, Chiapas	EU049712	EU049762	EU049786	EU049807	EU049736
Trigona fuscipennis	218	Costa Rica, Limón	EU049709	EU049759	EU049783	EU049804	EU049733
Trigona cf. *fuscipennis*	458	Brazil, São Paulo	EU049717	EU049767	EU049791	EU049812	EU049741
Trigona cf. *fuscipennis*	169	Peru, Madre de Díos	EU049706	EU049756	EU049779	EU049801	EU049729
Trigona guianae	024	Peru, Pasco	DQ790467	DQ813069	EU049770	DQ813226	EU049720
Trigona hyalinata	459	Brazil, São Paulo	EU049718	EU049768	EU049792	EU049813	EU049742
Trigona hypogea	380	Peru, San Martín	EU049714	EU049764	EU049788	EU049809	EU049738
Trigona lacteipennis	058	Peru, San Martín	EU049700	EU049750	EU049773	EU049795	EU049723
Trigona nigerrima	220	Costa Rica, Guanacaste	EU049711	EU049761	EU049785	EU049806	EU049735
Trigona pallens	061	Peru, San Martín	EU049702	EU049752	EU049775	EU049797	EU049725
Trigona cf. *recursa*	167	Peru, Madre de Díos	EU049704	EU049754	EU049777	EU049799	EU049727
Trigona recursa	446	Brazil, São Paulo	EU049715	EU049765	EU049789	EU049810	EU049739
Trigona silvestriana	187	Costa Rica, Guanacaste	EU049707	EU049757	EU049780	EU049802	EU049730
Trigona spinipes	447	Brazil, São Paulo	EU049716	EU049766	EU049790	EU049811	EU049740
Trigona truculenta	212	Peru, Madre de Díos	EU049708	EU049758	EU049782	EU049803	EU049732
Trigona williana	371	Peru, Loreto	EU049713	EU049763	EU049787	EU049808	EU049737
Cephalotrigona sp.n.	074	Peru, San Martín	DQ790465	DQ813033	DQ813110	DQ813188	EU049743
Geotrigona fulvohirta	007	Peru, San Martín	DQ790466	DQ813039	DQ813116	DQ813194	EU049744
Melipona grandis	017	Peru, San Martín	DQ790458	DQ813051	DQ813130	DQ813208	EU049745
Partamona auripennis	013	Peru, San Martín	DQ790463	DQ813056	DQ813135	DQ813213	EU049746
Scaptotrigona polysticta	010	Peru, San Martín	DQ790461	DQ813063	DQ813142	DQ813220	EU049747

into exon and intron regions to account for rate variation among gene regions. Substitution models for partitions were determined on the basis of the Akaike information criterion (AIC) in Modeltest version 3.7 (Posada and Crandall, 1998). The model parameters used for each gene partition were: 16S (TVM+I+G); opsin intron (TIM), opsin exon (TVM+I+G); ArgK intron (K81uf), ArgK exon (TrNef+I); EF-1α intron (HKY+I), EF-1α exon (TVM+I); 28S (HKY+I). To examine character homogeneity among the different gene partitions, we applied the partition homogeneity (Incongruence Length Difference, ILD) test (Farris et al., 1995) implemented in PAUP* (Swofford, 2002) (heuristic search, 100 replicates, 10 random additions of taxa per replicate, TBR branch swapping, retaining 500 trees per replicate). Four replicate independent Bayesian analyses (nine million generations, four chains, mixed models, flat priors, trees sampled every 100 generations) were run for a combined gene dataset. Each of the four majority-rule consensus trees from the replicate runs were examined for convergence and stationarity in Tracer 1.2. (Rambaut and Drummond, 2005). Trees estimated prior to stationarity were discarded (25%) and trees remaining after convergence from the replicate runs were combined into a single majority-rule consensus tree. Bayesian posterior probability values represent the proportion of trees from the Markov Chain Monte Carlo (MCMC) samples that contain a given node, and are interpreted as the probability that a node is correct given the data and the underlying model. To compare results obtained from Bayesian analysis, a heuristic search was implemented under the maximum likelihood (ML) criterion in PAUP* (200 replicates, model TVM+I+G), applying parameters estimated in Modeltest. Nonparametric ML bootstrapping (1000 replicates, GTR model, p-invar = 0.49) was implemented in PHYML version 2.4.4 (Guindon and Gascuel, 2003). To compare model-based methods with results under the parsimony (MP) criterion, we implemented parsimony analysis (heuristic search, 10 000 random additions, TBR branch swapping, all characters of equal weight) and parsimony bootstrap (heuristic search, 1000 replicates, 100 random additions per replicate) in PAUP*.

2.3. Evolution of behavioral traits

The evolutionary trajectories of nest characters were inferred by mapping them onto the Bayesian phylogeny using MacClade version 4.08 (Maddison and Maddison, 1992). Ancestral states were inferred from MacClade, including the outgroups, using DELTRAN, ACCTRAN, and unambiguous ("all most parsimonious states") optimizations to cover the full range of equally parsimonious solutions. For each of the mapped characters from the ingroup, we tested for phylogenetic association on the phylogeny by computing the probability distribution of the number of state changes when character distributions are randomized (i.e., whether characters evolved on the phylogeny in a pattern that was significantly different from a random pattern) (Maddison and Slatkin, 1991). This was done by randomly reshuffling each character 1 000 times on the Bayesian phylogeny and comparing the number of steps of the original tree to that of the null distribution. The set of nest characters used was based on field observations of one of the authors (Camargo, Figs. 1–18), supplemented by literature reports. While most observations were made on multiple nests of *Trigona s.s.* taxa (a total of approximately 200 detailed field observations), these may not fully represent the diversity within each species. The character states for each taxon are listed in Table III. Each of the 15 nest characters, including a brief discussion of coding when necessary are defined below.

2.4. Characters

Character 1: Nesting substrate. [0] Hollow tree (Figs. 12, 13); [1] Exposed (Figs. 2–7); [2] Association with termite colony (Figs. 8, 11, 14, 15, 17, 18); [3] Association with ant or wasp colony (Fig. 9); [4] Subterranean (Fig. 10); [5] Semi-exposed in tree cavity (Fig. 1). Explanation: 1.0. The nest is located inside a hollow tree trunk, with only a small orifice for passage of bees, and no association with colonies of other social insects. 1.1. Exposed or free nests are constructed around tree branches (Figs. 4, 6, 7), or attached to palms (Fig. 3), tree trunks (Fig. 2), house walls (Fig. 5), rocks (hillsides), etc. The nest habits reported for *T. amalthea* are only those referred in the original description of the species (Olivier, 1789), and those of Provancher (1888, p. 345) and Myers (1935) that studied *T. trinidadensis* (= *T. amalthea*) from Trinidad. 1.2. Nests of several species of epigeal termites may be utilized by stingless bees: inside tree trunks (*T. pallens*), attached to lianas (*T. fuscipennis* group), exposed and attached to tree trunks and branches. Not included here are the cavities of hypogeal

Figures 1–9. *Trigona* nests from Brazil. (**1**) *Trigona truculenta*, partially inside trunk of "Apuí" (*Ficus* sp., Moraceae), Ilha Grande (0° 31' 12"S, 65° 04' 37"W), rio Negro, AM, 16.VII.1999; (**2**) *T. amazonensis*, on trunk of "Samaúma" (*Ceiba* sp., Bombacaceae), rio Marauia, rio Negro, AM, 1.VII.1980; (**3**) *T. branneri* on trunk of "Tucumã" palm (*Astrocaryum* sp., Arecaceae), Bacururú (3° 45'S, 66° 11'W), rio Juruá, AM, 1.VIII.1993; (**4**) *T. spinipes*, Nina Rodrigues, MA, 17.VII.1982; (**5**) *T. hyalinata*, Porto Nacional, TO, VII.1994; (**6**) *T. dallatorreana* in tree top, Caborini (3° 7'S, 64° 47'W), rio Japurá, AM, 23.VIII.1993; (**7**) *T. dallatorreana*, Nazaré (0° 31'S, 65° 04'W), rio Negro, AM, 15.VII.1999; (**8**) *T. cilipes*, in termite nest, Carixeno (0° 20' 58"S, 65° 59' 47"W), rio Negro, AM, 8.VII.1999; (**9**) *T. lacteipennis*, in ant nest (*Azteca* sp., Formicidae), Samaúma (0° 26'S, 64° 45' 35" N), rio Negro, AM, 17.VII.1999.

Figures 10–18. *Trigona* nests from Brazil. (**10**) *T*. *recursa*, subterranean nest entrance, rio Ipixuna, Purus, AM, 20.I.1986; (**11**) *T*. *pallens*, nest entrance, located in termite nest inside hollow trunk, Tefé, AM, 28.I.1977; (**12**,**13**) *T*. *hypogea*, in hollow trunk, Itaituba, PA, 19.I.1979; (**14**) *T*. *guianae*, in epigeous termite nest, Arimã, rio Purus, AM, 15.II.1986; (**15**). *T*. aff. *fuscipennis*, in arboreal termite nest, Japurá, rio Purus, AM, 29.I.1986; (**16**) *T*. *dallatorreana*, showing the scutellum in the lower part of the nest, Paraíso (2° 09'S, 65° 05'W), rio Japurá, AM, 21.VIII.1993; (**17**) *T*. *chanchamayoensis*, showing irregular combs and permanent pillars, Tauari, rio Tapajós, PA, 30.I.1979; (**18**) *T*. *cilipes*, in termite nest, Carixeno (0° 20' 58"S, 65° 59' 47"W), rio Negro, AM, 8.VII.1999.

Table III. Character matrix for stingless bee nest architecture and behavior. For an explanation of the different characters and their character states, refer to material and methods.

	01	02	03	04	05	06	07	08	09	10	11	12	13	14	15
Trigona chanchamayoensis (016)	2	0	1	0	0/1	1	1	1	0	0	0	1	0	0?	1
T. pallens (061)	2	0	1	0	1	1	1	1	0	0	0	0	1	0?	0
T. guianae (024)	4/2	0	1	0/2	0	1	1	1	0	0	0	0?	0?	0?	1
T. fulviventris (299) (Panama) #	4	0	1	0	0	1	1	1	0	0	0	0	1	0?	1
T. williana (371)	0	0/1	1	1	0	1	1	1	0	0	0	1	1	0?	1
T. cilipes (045)	2	0	1	0	1	1	0	0	0	0	0	0	0	0	1
T. lacteipennis (058)	3	0	1	0	0	1	0	0	0	0	0	0	1	0	1
T. amalthea (052)	1	1/2	2	1	1	?	0	0	0	?	?	1	1	0?	0
T. truculenta (212)	5	1	2	1	1	1	0	0	0	?	0	1	?	?	0
T. silvestriana (187) #	1	1/2	2	1	0	1	0	0	0	1	0	1	1	0?	0
T. amazonensis (200)	1	1	2	1	1	1	0	0	0	1	0	1	0?	1	0
T. hyalinata (459)	1	1	2	1	1	1	0	0	0	1?	0	1	0?	1	0
T. spinipes (447)	1	1/2	2	1	1	1	0	0	0	1	0	1	0?	1	0
T. corvina (219) #	1	1/2	2	1	0/1	1	0	0	0	1	0	1	1	1	0
T. nigerrima (220) #	1	1/2	2	1	0/1	1	0	0	0	1	0	1	0?	0	0
T. dallatorreana (076)	1	1	2	1	0/1	1	0	0	0	1	0	?	0?	?	1
T. albipennis (168)	2	1	1	1	1	0/1	1/0	0	0	0	0	1	0?	2?	0
T. fuscipennis (218) (Panama) #	2	1	1	1	1	1	1/0	1/0	0	0	0	1	0?	2?	0
T. cf. *fuscipennis* (169)	2	0/1	1	1?	1	0/1	1	0	0	0	0	1?	0?	2	0
T. cf. *fuscipennis* (458)	2	0/1	1	1?	1	0/1	1	0	0	0	0	1?	0?	2	0
T. crassipes (060)	0	0	1	0	0	1	0	0	0	0	0	0?	2	0	0
T. hypogea (380)	0	0	1	0	1	0	0	0	0	0	0	0?	2	0	1
T. cf. *recursa* (167)	4?	1?	1?	1?	0?	0/1?	0?	0?	0?	0?	1?	1?	1?	0?	1?
T. recursa (446)	4	1	1	1	0	0/1	0	0	0	0	1	1	1	0	1
Cephalotrigona sp.	0	0	0	0	2	0	0	0	1	1	0	0	1	0	1
Scaptotrigona polysticta	0	0	0/1	0	0	0	0	0	1	0	0	0	0	0	1
Partamona auripennis	2	2	0	2	2	0	0/1	1	1	0	0	0	0	0	0
Melipona grandis	0	2	0	2	2/1	0	0	0	1	0	0	1	1	0	0
*Geotrigona fulvohirta**	4	0	1	0	2	0	0	0	1	0	0	0	0	0	0

Distribution in Central America.

* Including data from nests of *G. mombuca*, *G. subterranea*, *G. mattogrossensis*, and *G. kraussi*.

termites facultatively used by species of the *T. fulviventris* and *T. recursa* groups (see character state 1.4). 1.3. Ant and wasp nests were coded together, since only a single species use them facultatively (*T. lacteipennis*, Fig. 9, Rasmussen, 2004). *Trigona chanchamayoensis* utilizes termite nests co-existing with *Camponotus* sp. (Formicidae), but was coded as termitophile (character state 2). 1.4. Subterranean, in chambers of active or abandoned hypogeal termite colonies, or in any other underground chamber, chiefly resulting from roots of dead trees (*T. guianae*, *T. recursa*, and others). 1.5. Semi-exposed in partially open and hollow tree cavities, leaving the nest partially exposed and sheltered (only *T. truculenta*, Fig. 1).

Character 2: Building material. [0] Only wax and resins; [1] Vegetal fibers and particles (also exine from bee excrement or fecal pollen), in addition to wax and resins; [2] Soil, in addition to wax and resins. Explanation: 2.0. Nest constructed of wax and vegetal resins, no evidence for the use of additional materials, or additional materials are used in small quantities. 2.1. Vegetal fibers or particles are used in the construction of the external involucrum, or at least in the nest entrance. Many species of *Trigona* collect decomposing vegetal particles and add them to the resin for use mainly in the entrance tube; some also chew and macerate buds and fresh leaves (several *Trigona* are known as pests of *Citrus* trees) to obtain fibers that are mixed chiefly with resins for construction of the external protective involucrum. Among the remaining stingless bees, only *Tetragona goettei* is known to gather decomposing wood fibers, and uses them in the construction of the entrance tube. 2.2. The extensive use of soil has only been observed in nests of *T. guianae*. Reports of soil in the external involucrum has been made for nests of *T. amalthea*, *T. spinipes*, *T. corvina*, *T. nigerrima*, and *T. silvestriana* (Myers, 1935; Nogueira-Neto, 1962; Wille and Michener, 1973). Animal excrement may also be included as construction material (character 12: coprophily). In the outgroups *Melipona* and *Partamona* soil is amply used; many species of *Melipona* use pure soil for nest entrance construction and with added resin in the batumen; *Partamona* use soil in the entrance and several other parts of the nest (cf. Camargo and Pedro, 2003b).

Character 3: External involucrum. [0] Absent; [1] Present, made of resin or cerumen (Figs. 13–15, 17); [2] Present, made of vegetal particles and resins (Fig. 16). Explanation: 3.0. In this character state we coded the species of *Melipona*, *Cephalotrigona*, and *Scaptotrigona*, whose nests are encased above and underneath by batumen plates composed of soil and resins (*Melipona*) or only resins, while the inner walls of the nest cavity receive only minor resin applications. 3.1. This character state involves the species of *Trigona s.s.* that construct nests in hollow trees or cavities, inside termite or ant colonies, or underground; the outer first sheath of the involucrum is generally thick and applied directly on the substrate; the second sheath, which may be absent in some nests, is connected to the first sheath through connective pillars of cerumen or resins and is usually constructed of resins, resins and wax, or with addition of soil or vegetal fibers. The external involucrum in the outgroup *Geotrigona* is constituted of two or more sheaths, forming longitudinal galleries between them. 3.2. This character state includes species that mainly construct exposed nests, and whose involucrum is composed of lamellar sheaths that are regular in form with added vegetal fibers and resins (sometimes with addition of soil, see character 2) and a composition similar to cardboard. The involucrum of *T. williana*, which constructs nests in tree cavities, occasionally contains of vegetal fibers collected from cattle excrement.

Character 4: Material used in nest entrance. [0] Resins or cerumen; [1] Resins and vegetal particles; [2] Soil with the addition of resins.

Character 5: Shape of nest entrance. [0] Long tube, longer than wide; [1] Short tube, wider than long, or very reduced; [2] Not forming a tube. Explanation: This character is difficult to code as the nest entrance is often unique for each species. Character state 5.2 include several dissimilar outgroup species without a tube, ranging from *Cephalotrigona* where the entrance is merely a small orifice free of ornamentation and contrasted by *Partamona* that constructs an entrance including slopes for landing and take-off of foragers (cf. Camargo and Pedro, 2003b).

Character 6: Brood involucrum. [0] Present; [1] Absent. Explanation: Brood involucrum is considered where there are one or more lamellar sheaths of cerumen surrounding the brood region, distinct from the external involucrum where the sheaths surrounds the whole nest, including provisioning pots (cf. character 6.3).

Character 7: Brood combs. [0] Regular horizontal (discoidal, forming regular overlapping layers) (Figs. 13, 16, 18); [1] Irregular horizontal (forming small irregularly distributed combs) (Figs. 14, 17).

Character 8: Support of internal nest structures. [0] Temporary pillars made of cerumen; [1] Permanent pillars (Fig. 17). Explanation: Permanent pillars or beams are constructed of resins, or resins and soil, and support both brood and pots. They are not replaced as the small temporary pillars (vertical) and connectives (more or less horizontal) made of cerumen. In the *T. fuscipennis* species group thick permanent pillars may occur across the brood, but they are made of cerumen.

Character 9: Provision pots. [0] Small and almost spherical (up to 2 cm in height) (Figs. 13); [1] Large and oval (height over 2 cm).

Character 10: Scutellum. [0] Absent (Figs. 13–15, 17, 18); [1] Present (Fig. 16). Explanation: The scutellum (Fig. 16) described originally by von Ihering (1903, 1930) consists of a compact mass of debris (mainly exines or bee excrement with pollen) located between the sheaths of the involucrum below or on the sides of the brood region (see Nogueira-Neto, 1962). It is a well-developed structure in the species of *Trigona* that build exposed nests. In the outgroup, the character is only known in *Cephalotrigona* which deposits the debris between the layers of the lower batumen, forming a compact block.

Character 11: Pots for deposition of excrement. [0] Absent; [1] Present. Explanation: The habit of constructing large and irregular pots in the inferior part of the nest for deposit of excrement collected by the bees is known only in the *T. recursa* species group. These debris deposits differ from the above described scutellum by being constituted of regular pots made of cerumen and composed mainly of mammalian excrement. The strong smell of skatole and carrion is characteristic of the nests of *T. recursa*.

Character 12: Coprophily (or skatophily). [0] Absent; [1] Present. Explanation: Besides *T. recursa* (cf. character 11), several other species collect excrement for different construction uses in the nest. *Trigona nigerrima*, for example, applies excrement to the external involucrum of the nest (cf. Wille and Michener, 1973), while other species apply it to the entrance tube.

Character 13: Necrophagy. [0] Absent; [1] Facultative; [2] Obligate. Explanation: Some species of stingless bees collect exudates or small pieces of meat from animal carcasses, including *Melipona grandis*, *M. compressipes*, *Oxytrigona tataira*, *Cephalotrigona capitata*, *Trigona pallens*, among others (cf. Schwarz, 1948, pages 106, 300, 475; Cornaby, 1974, page 61; Roubik, 1982; Baumgartner and Roubik, 1989; Camargo and Roubik, 1991). Obligate necrophagy (conversion of animal protein to food), however, is only known in the *T. crassipes* species group. *Trigona recursa* has also been observed intensively collecting meat from animal carcasses for application to their nest entrance and, apparently, deposit in the excrement pots (see character 11); it is possible that pieces of meat are also deposited in their honey pots, which is of a terrible flavor.

Character 14: Hemipterophily. [0] Absent; [1] Facultative; [2] Obligate. Explanation: The habit of some species of *Oxytrigona* and *Trigona* attend Hemiptera in exchange for sweet secretions (honeydew) has been known for long time. In general these interactions with free living hemipteroids have been interpreted as opportunistic or facultative (Schwarz, 1948, pp. 104–106). Obligate hemipterophily is only known in *Schwarzula coccidophila* tending *Cryptostigma* sp. (Coccoidea) inside the nest (Camargo and Pedro, 2002) and possibly also in the *T. fuscipennis* group which has been observed tending membracids year round in southeastern Brazil (Carvalho, 2004).

Character 15: Nest defense behavior. [0] Aggressive; [1] Non-aggressive. Explanation: Certain species of *Trigona s.s.* react in swarms at the least disturbance and violently attack and bite the intruder (e.g., *T. spinipes* and *T. amazonensis*), while other species are docile and will not bite or attack (e.g., *T. williana* and *T. hypogea*).

3. RESULTS

3.1. Phylogeny

As the partition homogeneity test did not reveal significant incongruence ($P = 1.00$) among the partitions, we grouped the data into a single partition for combined analysis. The combined dataset consisted of 3 556 aligned nucleotides for the five gene fragments: 579 aligned nucleotides (bp) of 16S; 595 bp of opsin, including one intron comprising 150 bp; 724 bp of ArgK containing an intron of 180 bp; 842 bp of EF-1α F2 copy, containing an intron of 273 bp; and 816 bp of 28S. The interspecific nucleotide difference varied among genes, 16S the most variable gene fragment ranged from 1.7% (*T. truculenta* and *T. silvestriana*) to 10.7% divergence, with a mean divergence of 6.6%.

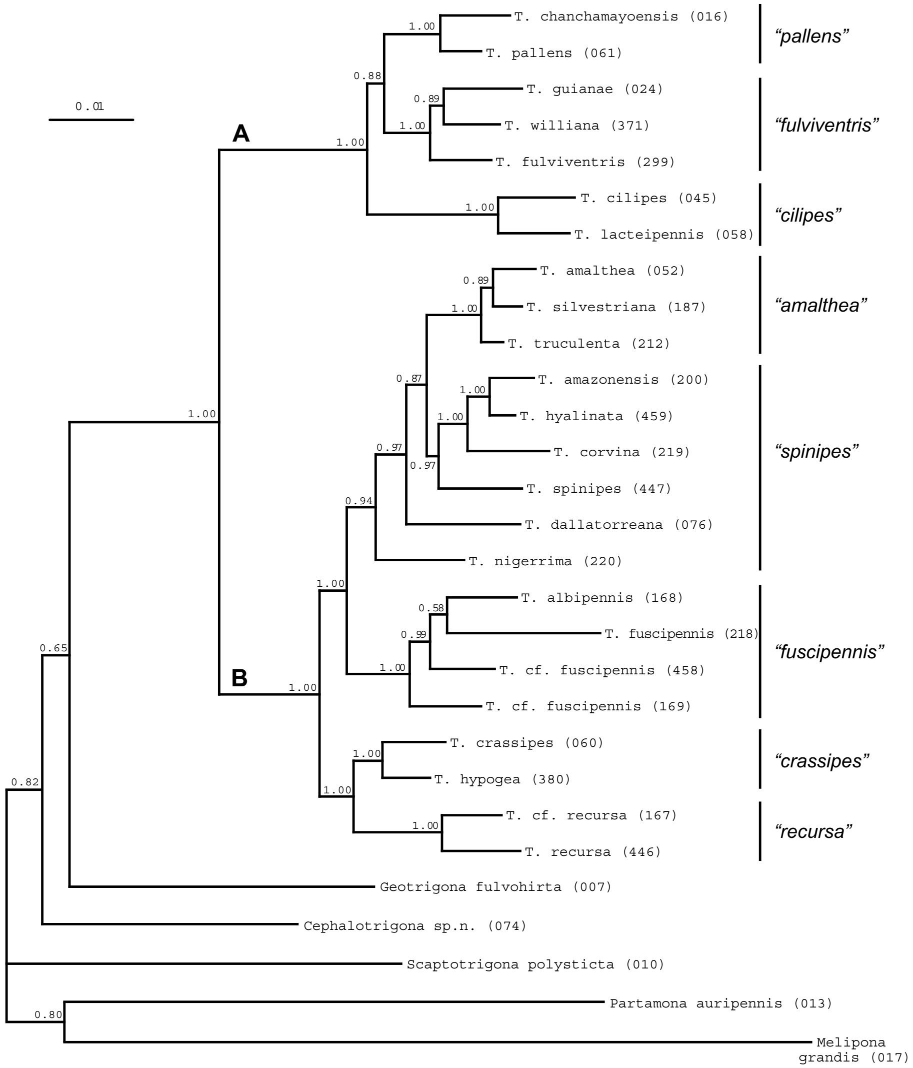

Figure 19. Phylogeny of *Trigona* estimated from Bayesian analysis of combined sequence data from five gene fragments (16S, opsin, EF-1α, ArgK, 28S). Values above the branches are Bayesian posterior probabilities. Species groups are indicated by vertical bars. Incongruence in the limitations of the species groups with morphology are only with the placement of *T. williana* in the *T. fulviventris* group while morphology placed it in the *T. pallens* group. The *T. spinipes* group is paraphyletic with respect to the *T. amalthea* species group. In parentheses, codes for terminal taxa (see Tab. II).

The combined Bayesian analysis resulted in a well-resolved phylogenetic hypothesis for a monophyletic *Trigona s.s.* (Fig. 19: PP = 1.00). Two distinct principal clades were recovered, both in the combined dataset (Fig. 19, clades labeled A and B, PP = 1.00/1.00) and within each individual gene fragment (figures not shown): 16S (PP = 1.00/1.00), opsin (PP = 0.99/0.82), ArgK (PP = 0.96/1.00), EF-1α (PP = 0.84/1.00), 28S (PP = 0.99/0.88). Minor clades (discussed below) were recovered in both the combined analyses and individual gene fragment analyses, except for 28S, which provided support only for the two

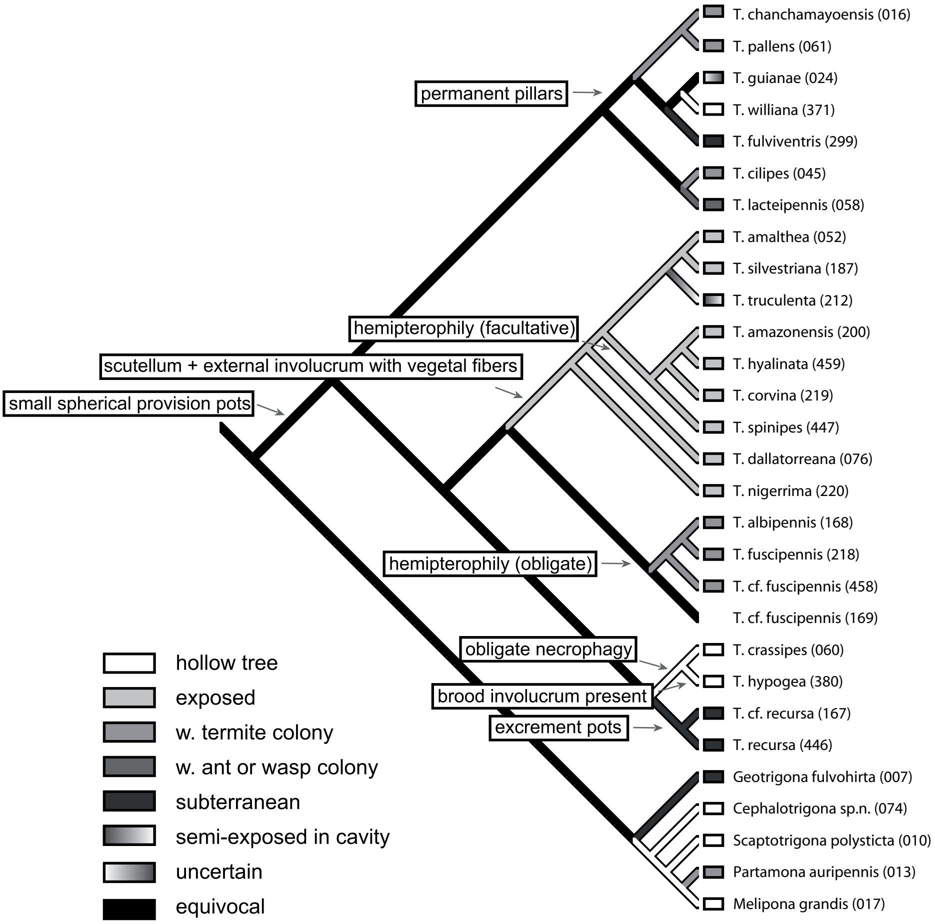

Figure 20. Optimization mapping of nest substrate on the *Trigona s.s.* phylogeny. Character states are indicated by the legend and shade of the branches. Additional derived characters are noted for the ingroup when restricted to a single clade. Outgroup characters were included for the optimization and for inferring ancestral state for the *Trigona s.s.* clade.

main clades. Seven of the species groups were recovered as monophyletic in the combined analysis: *T. amalthea* (PP = 1.00), *T. cilipes* (PP = 1.00), *T. crassipes* (PP = 1.00), *T. fulviventris* (PP = 1.00), *T. fuscipennis* (PP = 1.00), *T. pallens* (PP = 1.00), and *T. recursa* (PP = 1.00) (Fig. 19). Representatives for the *T. dimidiata* species group were not included and the *T. spinipes* (PP = 0.94) species group was paraphyletic with respect to the *T. amalthea* species group. ML and MP (Figures in online supplemental material) provided support for the major relationships inferred from Bayesian inference. Parsimony, however, resulted in three polytomies within species groups, and the Bayesian position of *T. truculenta* and *T. silvestriana* was reversed. Maximum likelihood reversed the position of two species pairs inferred from Bayesian and MP analyses (*T. truculenta* and *T. silvestriana*; *T. fulviventris* and *T. williana*), but was otherwise identical to the Bayesian tree.

3.2. Evolution of behavioral traits

The use of nesting substrate, presence of an external involucrum, presence of a brood

Table IV. Character sets; including the inferred ancestral state of *Trigona s.s.* from MacClade (DELTRAN and "all most parsimonious states", including the outgroups), the number of steps within the ingroup for each character on the Bayesian tree, and the *P*-value for a phylogenetic association of each character (excluding the outgroups).

Character	Inferred ancestral state	Steps	*P* value
1. Nesting substrate	equivocal	7	< 0.001*
2. Construction material	wax and resin (0)	2	< 0.001*
3. External involucrum	resin and cerumen (1)	1	< 0.001*
4. Nest entrance (material used)	resins or cerumen (0)	3	0.003 **
5. Nest entrance (shape)	equivocal	5	0.166
6. Brood involucrum	absent (1)	1	1
7. Brood combs	regular horizontal (0)	2	< 0.001*
8. Support of internal nest structures	temporary pillars (0)	1	< 0.001*
9. Provision pots	small spherical (0)	0	1
10. Scutellum	absent (0)	1	< 0.001*
11. Pots for deposition of excrement	absent (0)	1	0.022 **
12. Coprophily	absent (0)	4	0.110
13. Necrophagy	equivocal	7	0.115
14. Hemipterophily	absent (0)	2	< 0.001*
15. Nest defense behavior	non-aggressive (1)	4	< 0.001*

* *P* value < 0.001; ** *P* value < 0.05; a *P* value of 1 indicates that there is no variation in the ingroup.

involucrum, use of permanent pillars, size of provision pots, presence of scutellum, pots for deposition of excrement, hemipterophily, and obligate necrophagy are optimized onto the phylogeny in Figure 20. All characters mapped onto combined and individual trees are available in the online supplemental material. Ten of the 15 characters examined contained significant phylogenetic signal within the ingroup (Tab. IV), or showed significantly fewer steps than if the characters had been randomly assigned, including nest substrate, construction material for nest and nest entrance, support of internal nest structures, presence of external involucrum, scutellum, pots for deposition of excrement, and hemipterophily, as well as the shape of brood combs and nest defense behavior. Presence of a brood involucrum and the size and shape of provision pots did not vary among the ingroup taxa (ingroup with no variation or only a single character state different from the remainder of the ingroup). Three characters exhibited no phylogenetic association: shape of nest entrance, coprophily, and necrophagy. Inferred ancestral character states are listed in Table IV. ACCTRAN and DELTRAN optimizations of character states gave contrasting interpretations only for nesting substrate and necrophagy, both with equivocal ancestral states under DELTRAN optimization, while termitophile and facultatively necrophageous under ACCTRAN.

4. DISCUSSION

With a large taxon sampling of the *Trigona s.s.*, including most of the nominal species and species groups, we have confirmed the presence of several monophyletic species groups (cf. Tab. I). The *T. spinipes* species group appears paraphyletic and the placement of *T. williana* is not congruent with the morphological and biological data (Fig. 19). Representatives of the *Trigona dimidiata* species group were not sampled, thus its phylogenetic position is uncertain. They may belong to the clade of species constructing exposed nests (*T. spinipes*-*T. amalthea* groups) as Schwarz (1948) reported an exposed nest of *T. dimidiata*. The inclusion of *T. dimidiata* with the other builders of exposed nests may alter the paraphyly of the *T. spinipes* species group, and we therefore maintain the use of all species groups from Table I until further data become available. The molecular data also fully corroborate morphology in separating the multiple morpho-species of *T. recursa* and *T. fuscipennis* into distinct biological species based on interspecific nucleotide differences. The least difference encountered was between a sister-species pair distributed on either side of the Andean mountain range: the Amazonian *T. amalthea* and *T. silvestriana* from western Ecuador to Central America. Further study may reveal if they correspond to a vicariance event from the Andean uplift, as proposed for other groups of stingless bees (Camargo and Moure, 1996; Camargo and Pedro, 2003b).

By demonstrating a clear phylogenetic association for ten nest characteristics, we establish their conservation across closely related species of *Trigona*. The choice of nesting substrate was associated with the phylogeny and comprises more variation within *Trigona s.s.* than in any other group of stingless bees. This may reflect a response to constraints posed by nest site limitations on stingless bees in general, thus favoring transitions for the use of other types of substrates. Roubik (2006) suggested that between 15 and 1 500 colonies of stingless bee nests may be encountered in a square kilometer of natural vegetation. Their local abundance in combination with the limitation of appropriate cavities for establishing nests (Brown and Albrecht, 2001), provides an advantage for taxa employing alternative nest substrates to the otherwise widespread tree cavity nesting (Wille and Michener, 1973). *Trigona s.s.* provides support for such selective constraints posed by nest site limitations, as the genus has been particularly successful in adapting to and occupying a range of nesting substrates with only three of the species here studied using the typical hollow trunks for nesting, the putative ancestral state for all of the stingless bees (Wille and Michener, 1973). Termite colonies are common in Neotropical forests (1 600 per km^2: Constantino, 1992) and often used as nest substrates for stingless bees (up to 12% of all the stingless bees: Wille and Michener, 1973). Termites are also an important substrate for *Trigona s.s.* where the use is restricted to the *T. cilipes*, *T. fuscipennis*, and *T. pallens* species groups, and where at least one species (*T. lacteipennis*) possess an apparently more derived condition of occupying the abundantly available ant and wasp nests as nesting substrates (Kempf, 1962; Kerr et al., 1967; Rasmussen, 2004).

The second main alternative to cavity nesting in *Trigona s.s.* is the construction of exposed nests. The species groups *T. amalthea* and *T. spinipes* both build exposed or partially exposed nests, a derived condition, and in Neotropical stingless bees otherwise found only in *Partamona helleri*, certain species of *Paratrigona*, and *Tetragonisca weyrauchi* (Schwarz, 1948; Wille and Michener, 1973; Camargo and Pedro, 2003b; Cortopassi-Laurino and Nogueira-Neto, 2003, and unpubl. data). An obvious advantage of building exposed nests, other than the limitation in availability of sufficiently large cavities, is the circumvention of size restriction otherwise limiting colony size when utilizing pre-existing cavities. Extremely large colony sizes of stingless bees are found chiefly in the *T. spinipes* species group of exposed nest builders, where nests of *T. amazonensis* may reach 3 meters in length and 1 meter across (Camargo, unpubl. data, Fig. 2).

The construction of exposed nests occurs only in species of *Trigona* that use both a vegetal-based external involucrum and build a structurally supportive scutellum (Fig. 16); exposed nests are otherwise not supported sufficiently by the brittle external involucrum constructed by species of *Trigona s.s.* and other Meliponini (Nogueira-Neto, 1962). Other phylogenetically associated characters of importance to the structural design and construction, include nest construction materials and the construction material used to fashion the nest entrance. Exposed nest builders (*T. spinipes*-*T. amalthea* groups), among others, use vegetal fibers, probably to improve resistance of the nest, without adding the weight of soil to their nest constructions. Phylogenetic association was also found with the presence of permanent pillars, otherwise only found in *Partamona* (Wille and Michener, 1973; Camargo and Pedro, 2003b), and with nest defense and arrangement of brood combs. The latter character is irregular horizontal in most of the species nesting inside the colonies of other social insects, although this appears to be variable throughout the stingless bees and may not generally be phylogenetically related (Michener, 1961).

Two notable characters largely independent of phylogenetic history are coprophily and necrophagy (including facultatively). The lack of association between the phylogeny and these characters suggests that they are evolutionary labile and that these materials are widely available as sources of construction materials, minerals, or proteins in the absence of sufficient floral energy resources. Only the *T. hypogea* group has made the full transition to obligate necrophagy, including modification of their hind tibial morphology by reduction of the corbicula, the pollen basket used for transport of pollen. The species instead rely on transport of carrion in the crop (Roubik, 1982) as described under that character state. In contrast to coprophily (excrement collected for different uses in the nest), the construction of storage pots containing excrement appear to be an autapomorphy for the *T. recursa* group, where the behaviorally innovative use of pots to store excrement for fermentation may constitute a heat source for the nest. Another phylogenetically independent character was the shape of the nest entrance, probably due to extensive interspecific plasticity in size and shape, as well as our difficulty in coding this character across the stingless bees.

Without better knowledge of the phylogenetic position of *Trigona s.s.* relative to the other Neotropical stingless bee genera, it must remain rather speculative how the ancestral *Trigona* nest may have looked. Our results indicate that it may have included an external involucrum constructed of resin and cerumen, regular horizontal brood without an involucrum surrounding the brood, small spherical provision pots, and none of the more complex features, such as presence of a supporting scutellum, pots for excrement fermentation, or hemipterophily. The ancestral state for nesting substrate is best regarded as equivocal, although ACCTRAN, which favors evolutionary reversals (Maddison and Maddison, 1992), supports an ancestral termite association and facultative necrophagy. Future contributions of additional biological data (especially comparative nesting biology and recruitment communication), morphology and a complete and well supported phylogeny of the approximately 60 species of *Trigona s.s.* will undoubtedly reveal more subtle trends in nesting behavior and the evolution of nest architecture of the stingless bees.

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Phylogénie moléculaire et évolution de l'architecture du nid et comportement chez *Trigona s.s.* (Hymenoptera : Apidae : Meliponini).

Abeille sans aiguillon / phylogénie moléculaire / comportement / nid / néotropical

Zusammenfassung – Eine molekulare Phylogenie und die Evolution von Nestarchitektur und Verhalten bei *Trigona s.s.* (Hymenoptera: Apidae: Meliponini). Stachellose Bienen zeichnet eine ausserordentliche inner- und zwischenartliche Variation in der Nestarchitektur aus. Wir beschreiben hier Nestarchitektur und Verhalten für die neotropische Stachellose Bienengattung *Trigona s.s.* Im Anschluss daran erstellen wir eine phylogenetische Hypothese aus der Kombination von Teilsequenzdaten für ein mitochondriales Gen (16S rRNA) und vier Kerngene (langwelliges Rhodopsin Kopie 1 (Opsin), Elongationsfaktor-1α Kopie F2, Argininkinase und 28S rRNA), und wir verwendeten diese in einem Test zur phylogenetischen Assoziation von 15 Nest- und Verhaltensmerkmalen. Die resultierende Phylogenie bestätigt die Vermutung mehrerer Artgruppen und teilt die Gattung *Trigona s.s.* in zwei Hauptgruppen auf. Wir fanden eine signifikante Assoziation zwischen der Phylogenie und den folgenden Merkmalen: Nestsubstrat, Vorkommen einer äusseren Nesthülle (Involucrum) und eines Scutellums, von Töpfen für die Ablagerung von Exkrementen, in der Form der Brutwaben, als auch für Hemipterophylie und Nestverteidigungsverhalten. Keine signifikante Assoziation zur Stammbaumtopologie zeigten die nachfolgenden Merkmale: Form des Nesteingangs, Koprophylie und Nekrophagie. Das Fehlen einer Assoziation einiger dieser Merkmale zur Phylogenie könnte zum einen auf ihrem generellen und offensichtlich adaptiven Wert beruhen (Koprophylie und Nekrophagie erschliessen leicht verfügbare Proteinquellen), oder zum anderen in der zwischenartlichen Plastizität in Grösse und Form des Nesteingangs liegen, so dass sie durch die Stammbaumtopologie weniger beschränkt sind. Die Vielfalt der Nestsubstrate, die von *Trigona s.s.* genutzt werden, könnte einer der Gründe für den Erfolg dieser Gattung sein: sie bauen offene Nester und nutzen auch häufig Termitennester als Nistorte. Innerhalb der *Trigona* Arten, die offenen Nester bauen, finden sich Arten, wie z.B. *T. amazonensis,* die Nester von bis zu 3 m Länge und 1 m Durchmesser bauen, und die damit die weltweit grössten Nester Stachelloser Bienen darstellen. Die Rekonstruktion der ursprünglichen Merkmalszustände weist darauf hin, dass *Trigona* ursprünglich ein Termiten- oder höhlenassoziiertes Nest hatte, mit einem äusseren Involucrum aus Harz und Cerumen, mit regulär angelegten horizontalen Brutwaben ohne inneres Involucrum um die Brut herum, mit kleinen, runden Vorratstöpfen und ohne komplexere Nestmerkmale, wie z.B. einem stützenden Scutellum, Töpfen für die Fermentierung von Exkrementen, oder Hemipterophylie.

Molekulare Phylogenie / Neotropisch / Stachellose Biene / Verhalten / Nest

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Online Material

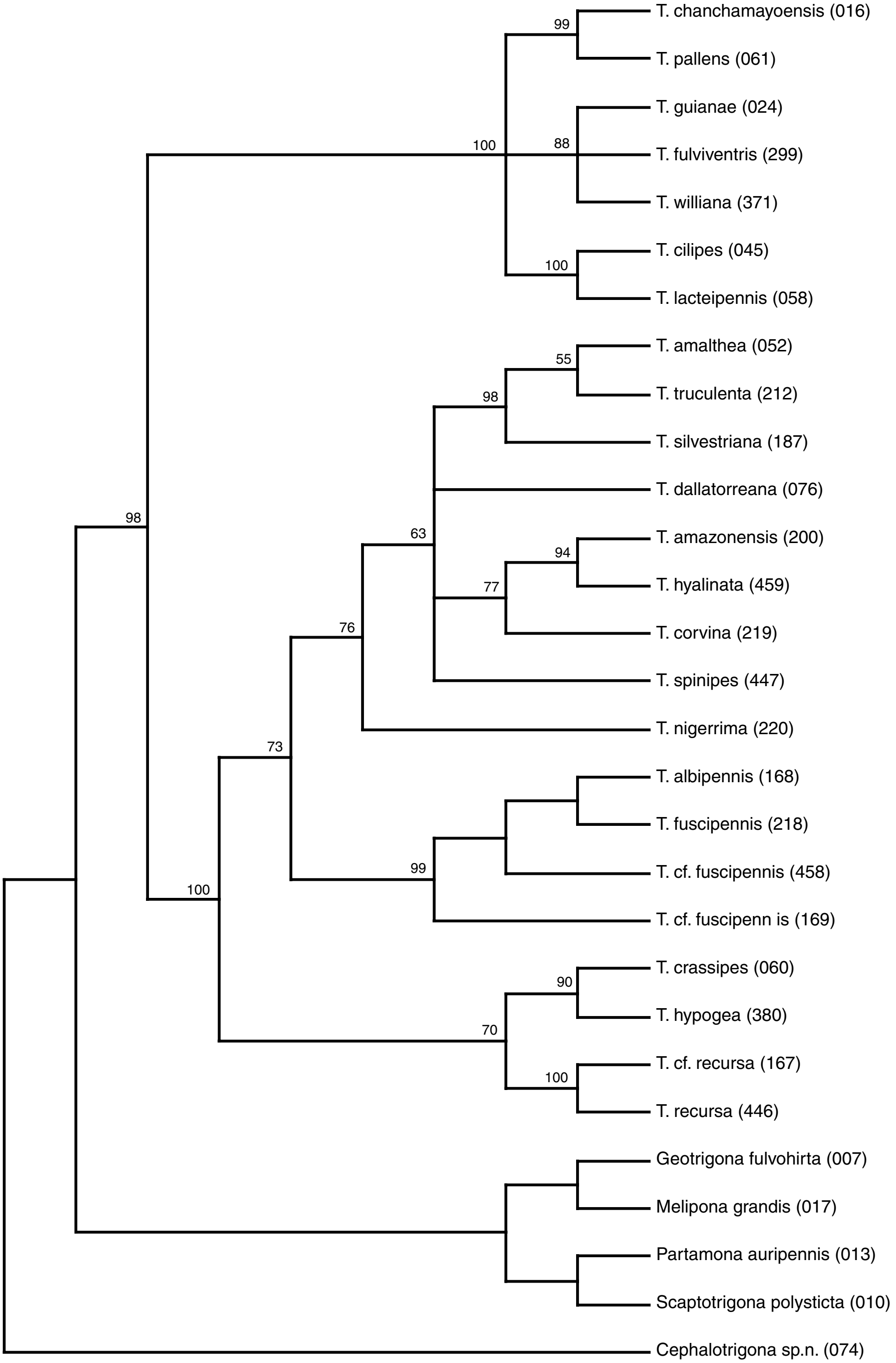

Figure 1. Phylogeny of *Trigona* (strict consensus of 9 trees) estimated from Maximum Parsimony analysis of combined sequence data from five gene fragments (16S, opsin, EF-1α, ArgK, 28S). Tree length (TL) = 973, consistency index (CI) = 0.57, retention index (RI) = 0.61. Values above the branches are bootstrap values $\geqslant 50$.

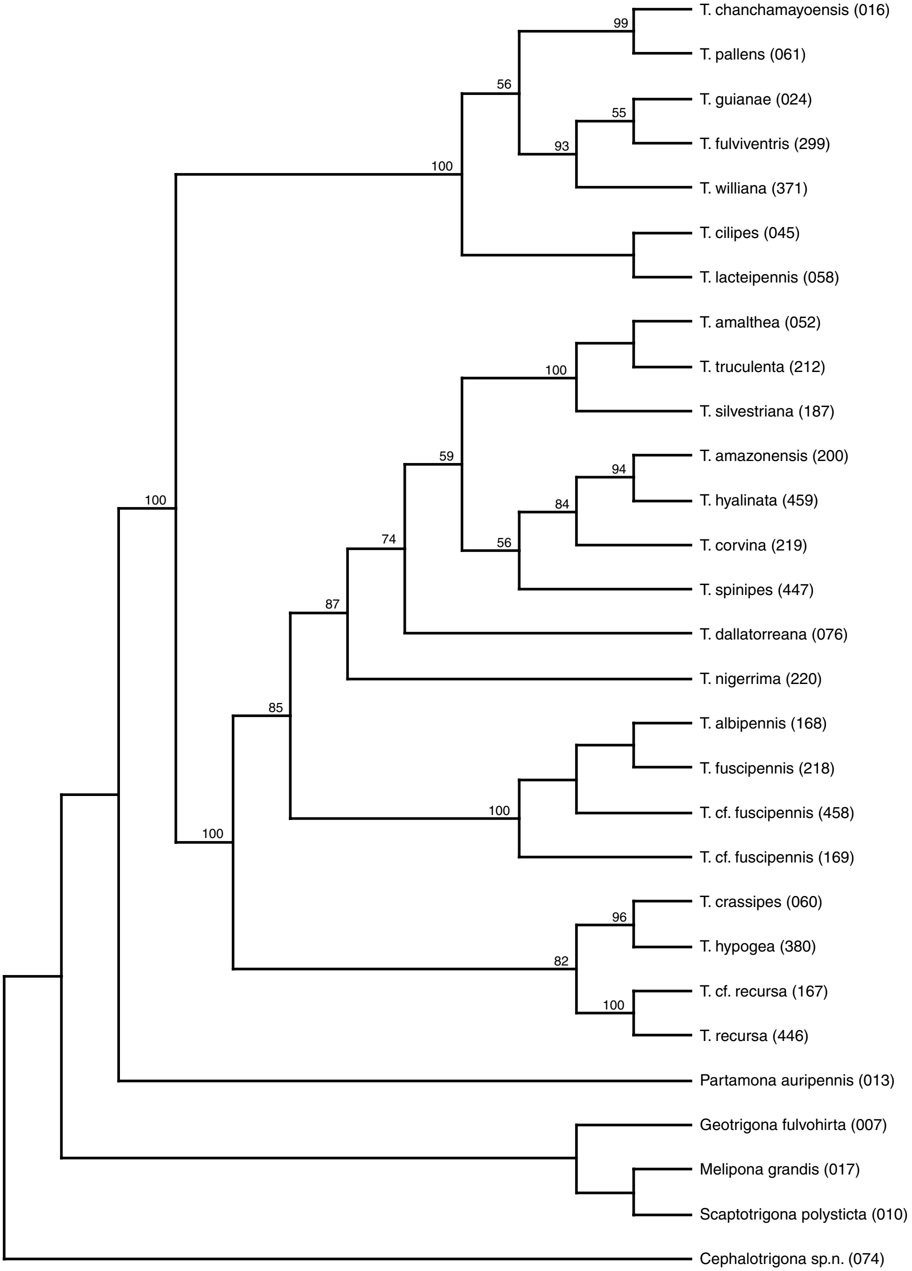

Figure 2. Phylogeny of *Trigona* estimated from Maximum Likelihood analysis of combined sequence data from five gene fragments (16S, opsin, EF-1α, ArgK, 28S). –Ln likelihood = 10 099.21316. Values above the branches are bootstrap values.

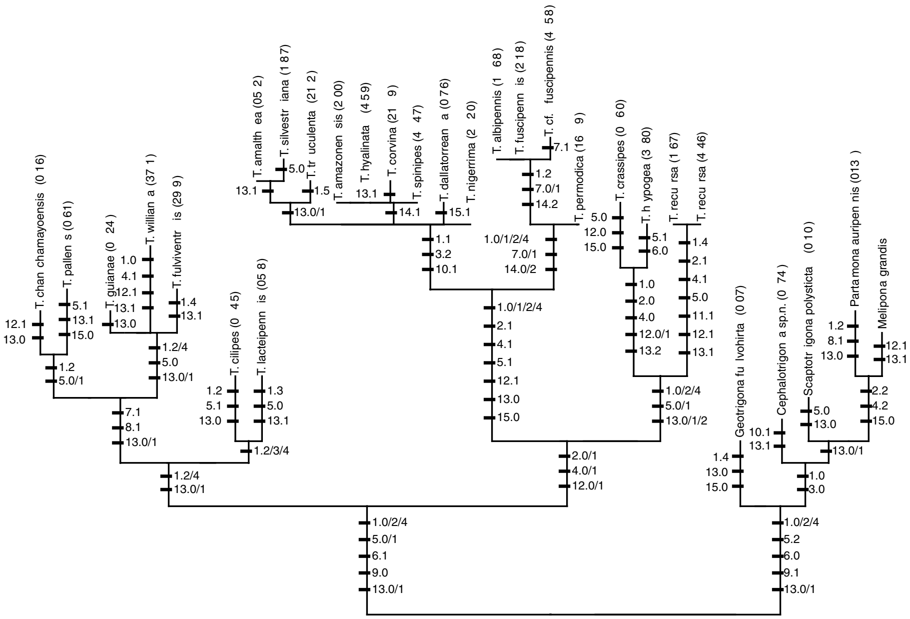

Figure 3. Optimization of all character changes onto the Bayesian phylogeny of *Trigona*.

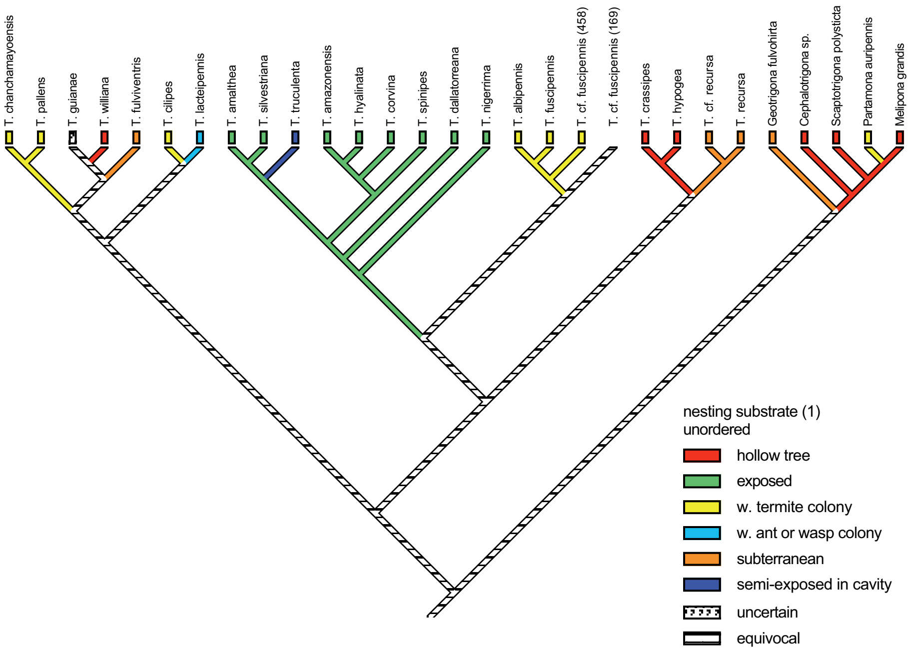

Figure 4. Optimization mapping of nest architectural and behavioral traits on the *Trigona s.s.* phylogeny. Character states are indicated by the legend and shade of the branches. Outgroup characters were included for the optimization and for inferring ancestral state for the *Trigona s.s.* clade.

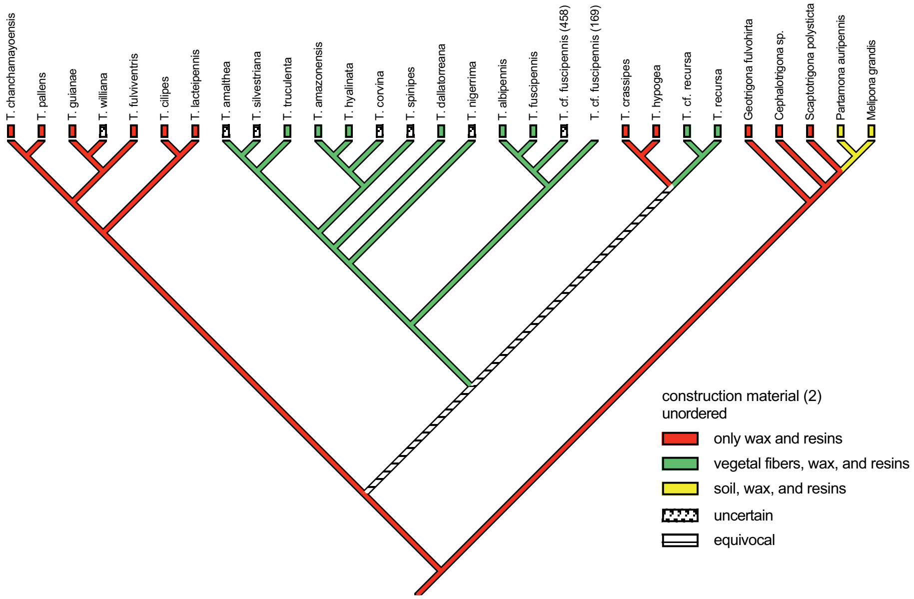

Figure 5. Optimization mapping of nest architectural and behavioral traits on the *Trigona s.s.* phylogeny. Character states are indicated by the legend and shade of the branches. Outgroup characters were included for the optimization and for inferring ancestral state for the *Trigona s.s.* clade.

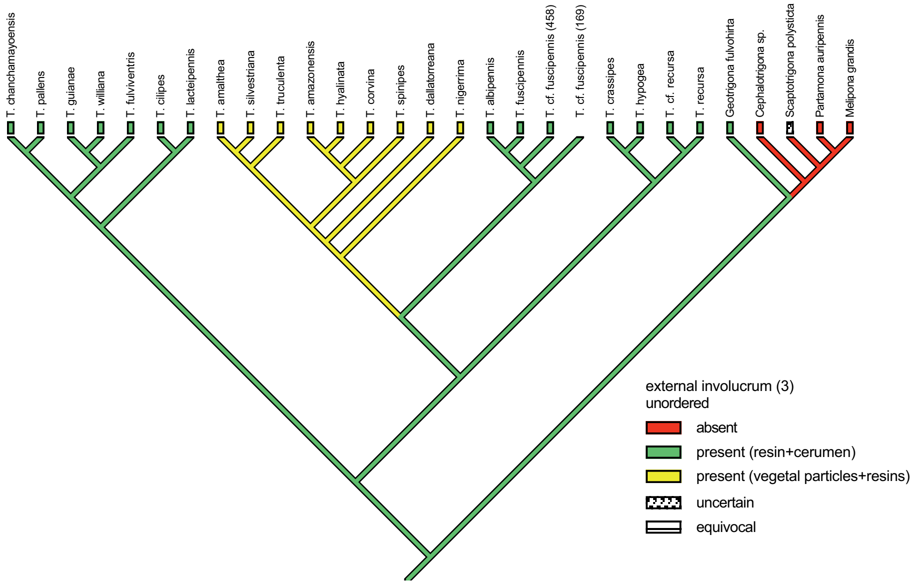

Figure 6. Optimization mapping of nest architectural and behavioral traits on the *Trigona s.s.* phylogeny. Character states are indicated by the legend and shade of the branches. Outgroup characters were included for the optimization and for inferring ancestral state for the *Trigona s.s.* clade.

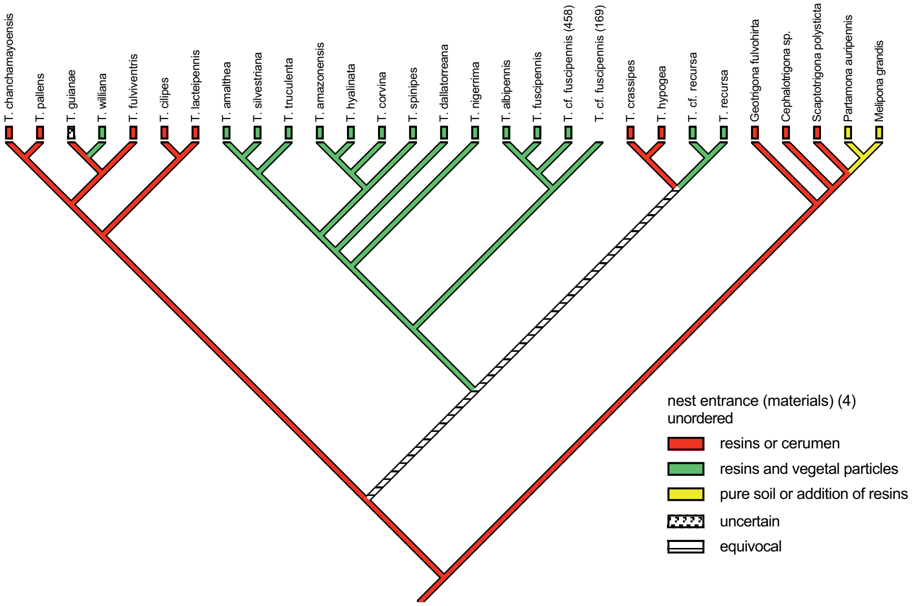

Figure 7. Optimization mapping of nest architectural and behavioral traits on the *Trigona s.s.* phylogeny. Character states are indicated by the legend and shade of the branches. Outgroup characters were included for the optimization and for inferring ancestral state for the *Trigona s.s.* clade.

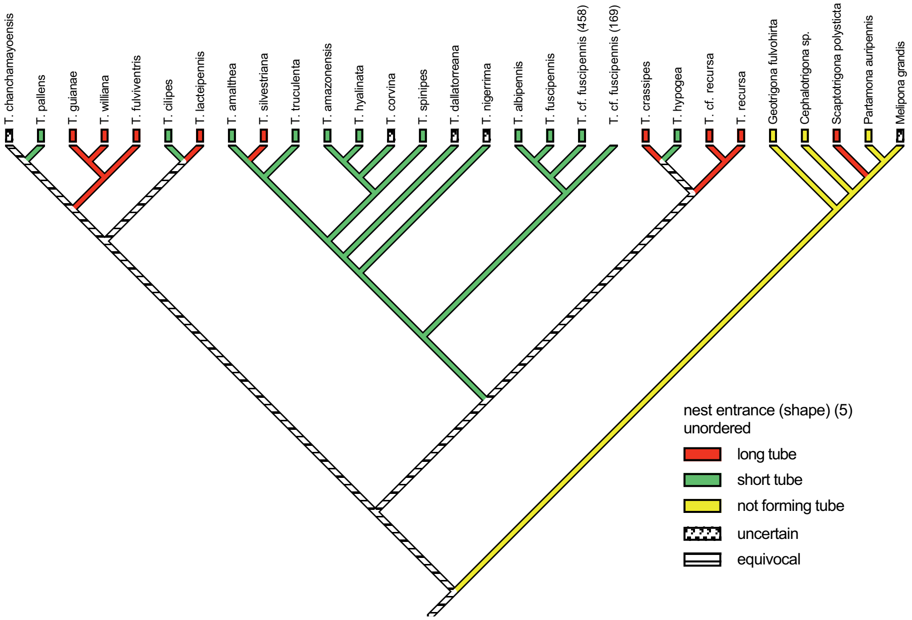

Figure 8. Optimization mapping of nest architectural and behavioral traits on the *Trigona s.s.* phylogeny. Character states are indicated by the legend and shade of the branches. Outgroup characters were included for the optimization and for inferring ancestral state for the *Trigona s.s.* clade.

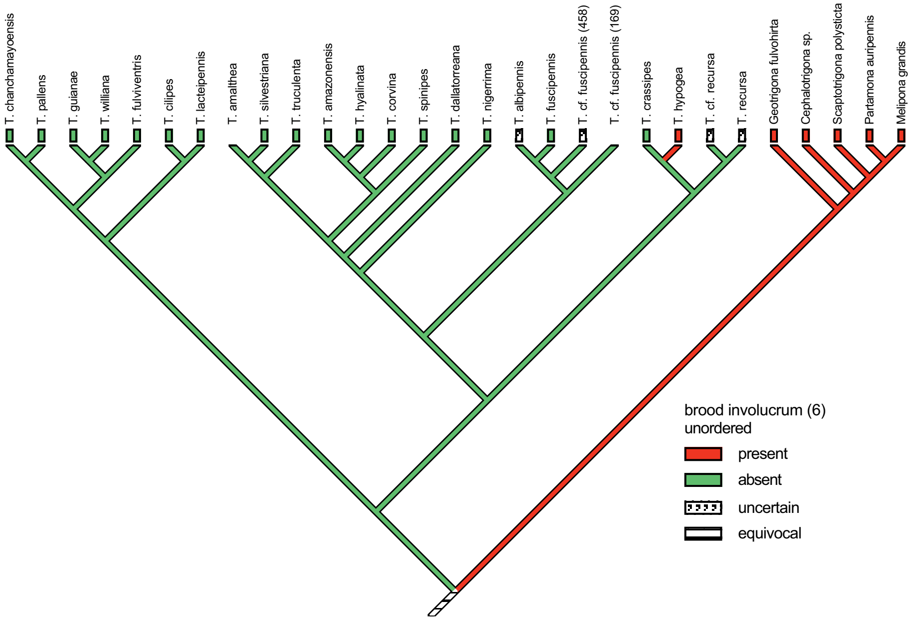

Figure 9. Optimization mapping of nest architectural and behavioral traits on the *Trigona s.s.* phylogeny. Character states are indicated by the legend and shade of the branches. Outgroup characters were included for the optimization and for inferring ancestral state for the *Trigona s.s.* clade.

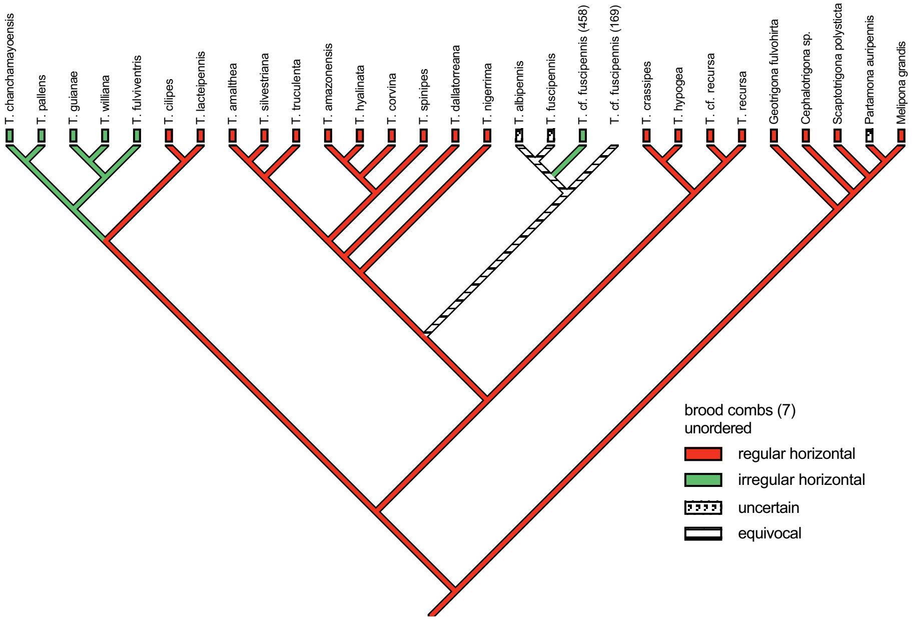

Figure 10. Optimization mapping of nest architectural and behavioral traits on the *Trigona s.s.* phylogeny. Character states are indicated by the legend and shade of the branches. Outgroup characters were included for the optimization and for inferring ancestral state for the *Trigona s.s.* clade.

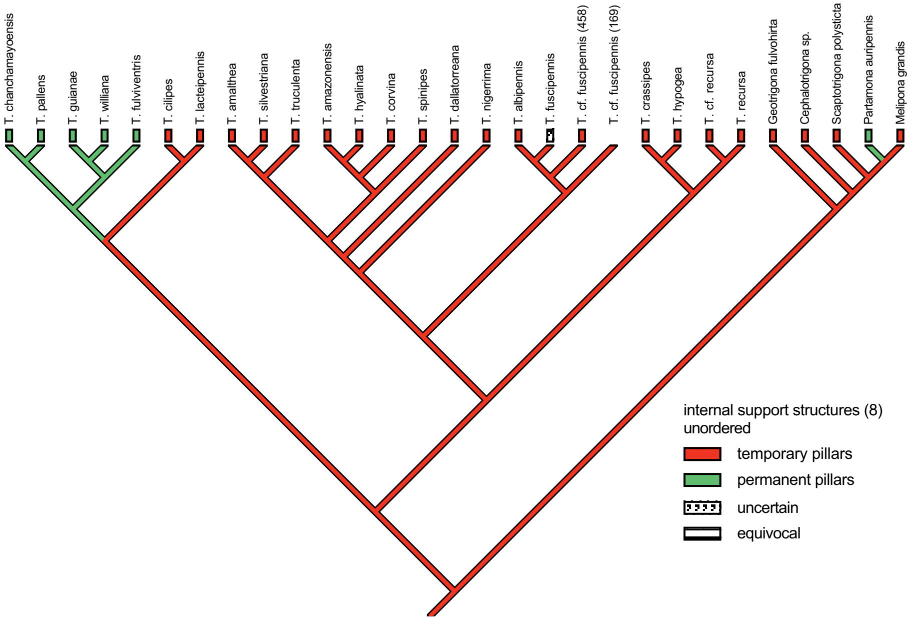

Figure 11. Optimization mapping of nest architectural and behavioral traits on the *Trigona s.s.* phylogeny. Character states are indicated by the legend and shade of the branches. Outgroup characters were included for the optimization and for inferring ancestral state for the *Trigona s.s.* clade.

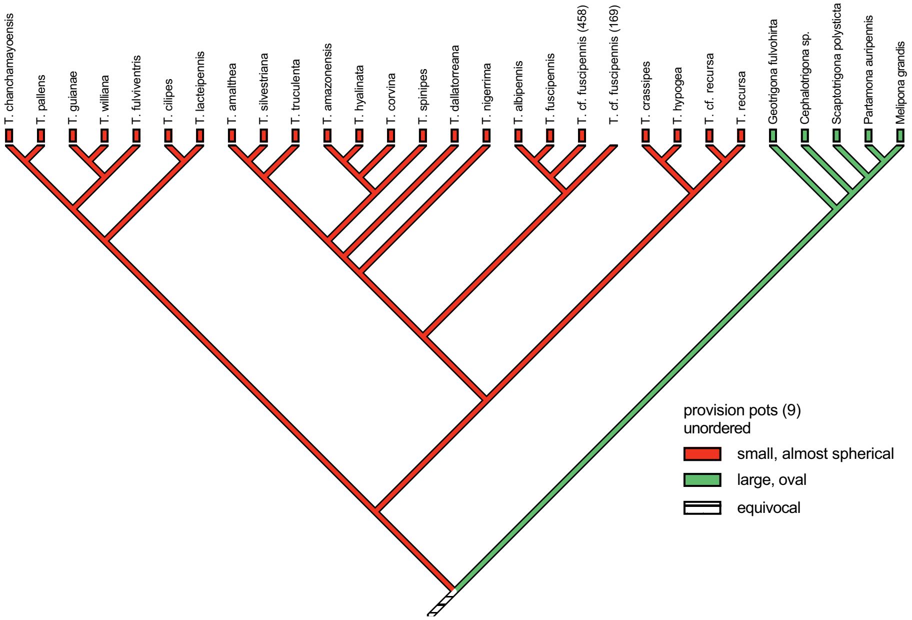

Figure 12. Optimization mapping of nest architectural and behavioral traits on the *Trigona s.s.* phylogeny. Character states are indicated by the legend and shade of the branches. Outgroup characters were included for the optimization and for inferring ancestral state for the *Trigona s.s.* clade.

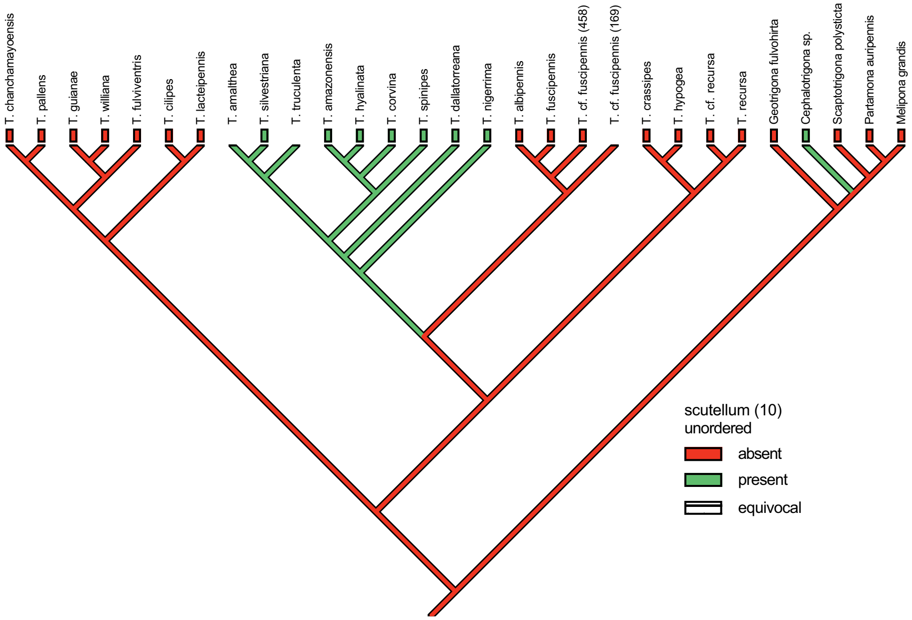

Figure 13. Optimization mapping of nest architectural and behavioral traits on the *Trigona s.s.* phylogeny. Character states are indicated by the legend and shade of the branches. Outgroup characters were included for the optimization and for inferring ancestral state for the *Trigona s.s.* clade.

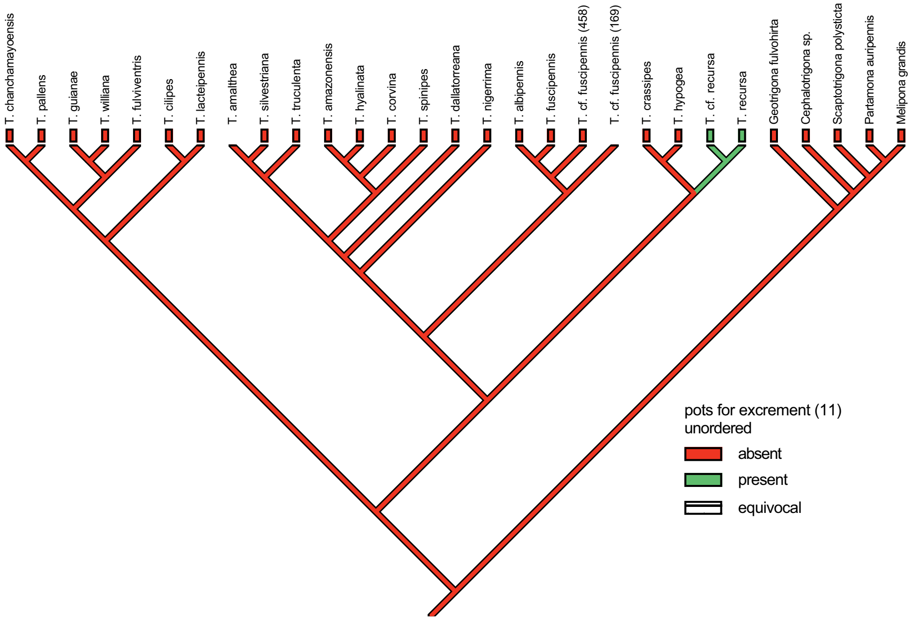

Figure 14. Optimization mapping of nest architectural and behavioral traits on the *Trigona s.s.* phylogeny. Character states are indicated by the legend and shade of the branches. Outgroup characters were included for the optimization and for inferring ancestral state for the *Trigona s.s.* clade.

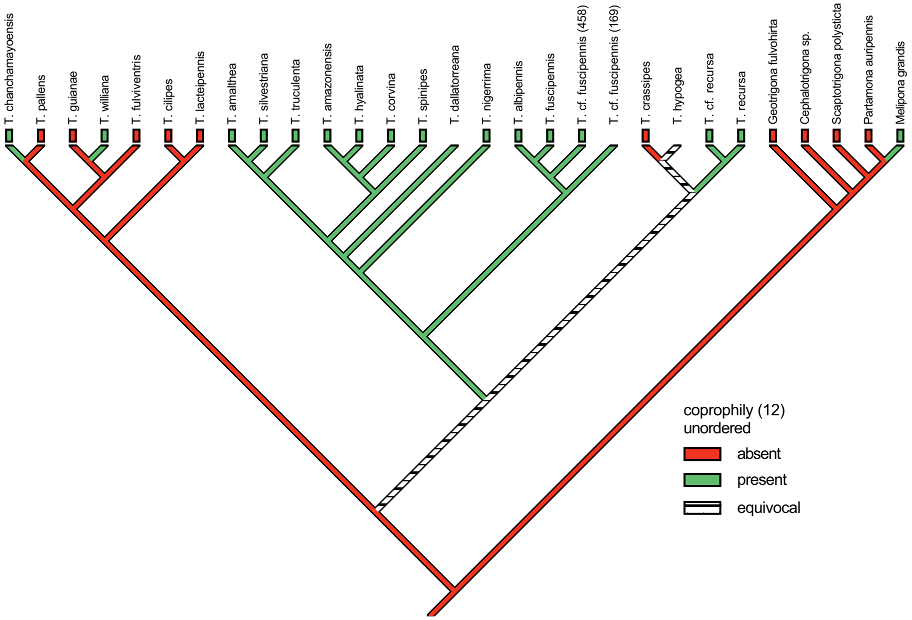

Figure 15. Optimization mapping of nest architectural and behavioral traits on the *Trigona s.s.* phylogeny. Character states are indicated by the legend and shade of the branches. Outgroup characters were included for the optimization and for inferring ancestral state for the *Trigona s.s.* clade.

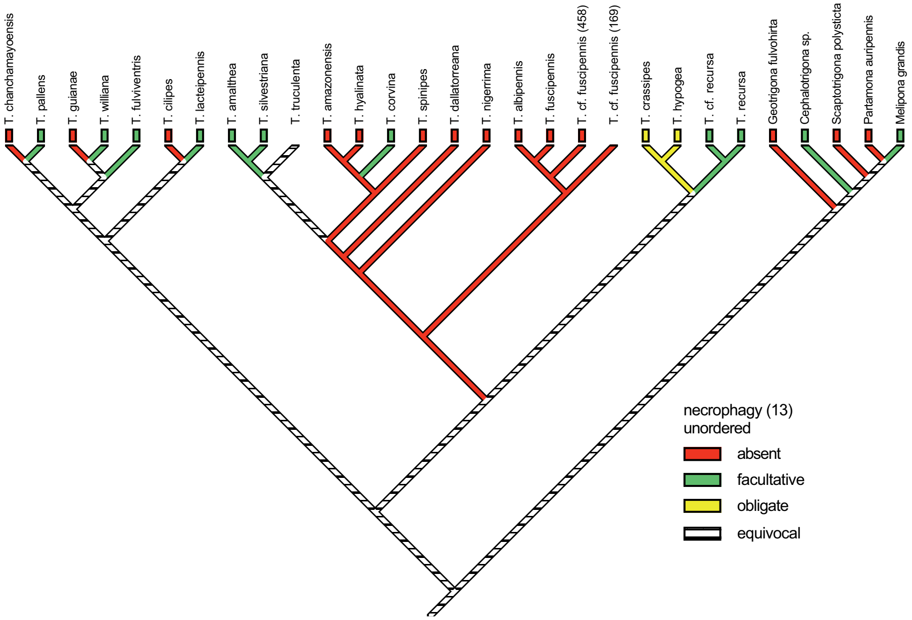

Figure 16. Optimization mapping of nest architectural and behavioral traits on the *Trigona s.s.* phylogeny. Character states are indicated by the legend and shade of the branches. Outgroup characters were included for the optimization and for inferring ancestral state for the *Trigona s.s.* clade.

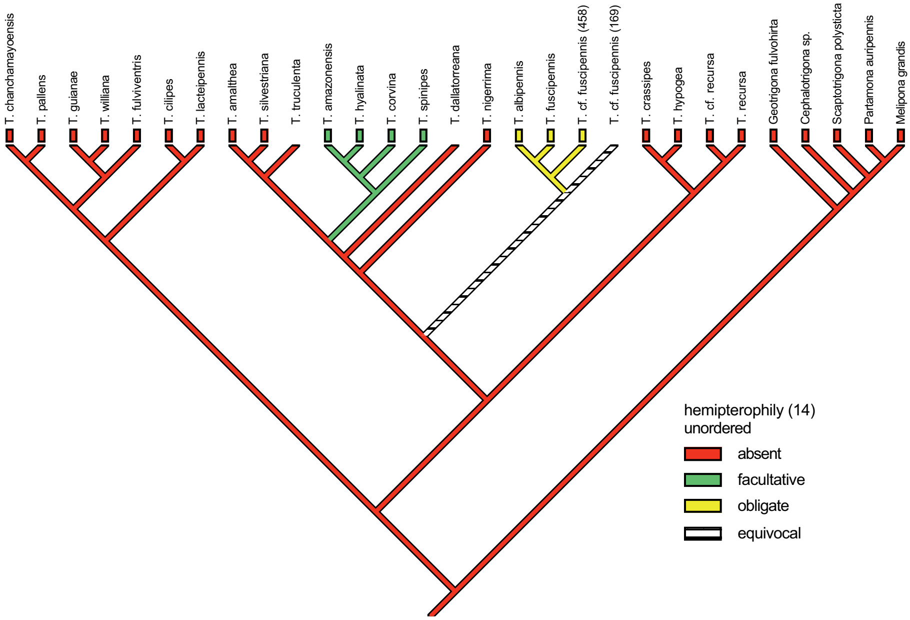

Figure 17. Optimization mapping of nest architectural and behavioral traits on the *Trigona s.s.* phylogeny. Character states are indicated by the legend and shade of the branches. Outgroup characters were included for the optimization and for inferring ancestral state for the *Trigona s.s.* clade.

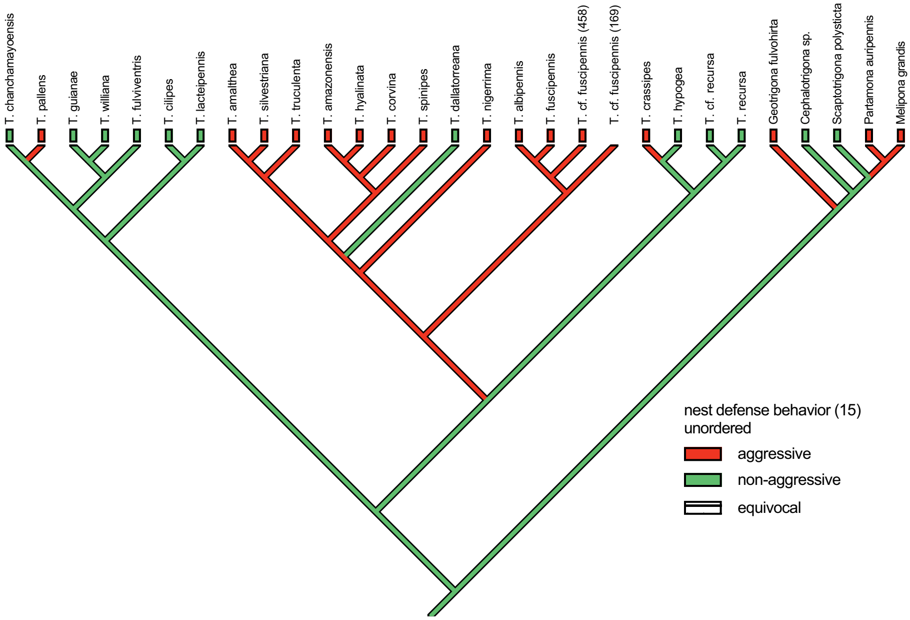

Figure 18. Optimization mapping of nest architectural and behavioral traits on the *Trigona s.s.* phylogeny. Character states are indicated by the legend and shade of the branches. Outgroup characters were included for the optimization and for inferring ancestral state for the *Trigona s.s.* clade.