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A molecular phylogeny of the Old World stingless bees (Hymenoptera: Apidae: Meliponini) and the non-monophyly of the large genus *Trigona*

CLAUS RASMUSSEN and SYDNEY A. CAMERON
Department of Entomology, University of Illinois, Urbana, Illinois, U.S.A.

Abstract. We examined the inter- and infrageneric relationships of Old World Meliponini with a near-complete sampling of supra-specific taxa. DNA sequences for the taxa were collected from four genes (mitochondrial 16S rRNA, nuclear long-wavelength rhodopsin copy 1 (opsin), elongation factor-1α copy F2 and arginine kinase). Additional sampling of New World taxa indicated that *Trigona sensu lato* is not monophyletic: *Trigona* from the Indo-Malayan/Australasian Regions forms a large clade distantly related to the Neotropical *Trigona*. A separate clade comprises the Afrotropical meliponines, and includes the 'minute' species found in the Afrotropical, Indo-Malayan and Australasian Regions. The Neotropical genus *Melipona*, by contrast with previous investigations, is not the sister lineage to the remaining stingless bees, but falls within the strongly supported Neotropical clade. These results constitute the framework for a revised classification and ongoing biological investigations of Meliponini. A single taxonomic change, *Heterotrigona bakeri* **stat.n.**, is proposed on the basis of sequence divergence.

Introduction

Stingless bees (Meliponini) are by far the most diverse, morphologically and behaviourally, of the eusocial corbiculate bees (Apini, Bombini, Meliponini) (Michener, 2000). They have also enjoyed a long history of discovery and description by naturalists and scholars during explorations of the tropical regions of the world (e.g. Bennet, 1831; Bates, 1863; Schwarz, 1948; Ruiz, 1998). Facets of their diversity are evident in their social organization, systems of communication, nest architecture and reproductive behaviour. Their perennial colonies range in size from fewer than 100 to tens of thousands of workers (Roubik, 1989; Drumond *et al*., 1997; Michener, 2000) and usually contain a single queen (Velthuis *et al*., 2001). Meliponines utilize diverse and elaborate communication systems with well-developed recruitment mechanisms that include scent-marking (Lindauer & Kerr, 1958, 1960; Hubbell & Johnson, 1978; Nieh, 1998, 1999; Nieh & Roubik, 1998; Nieh *et al*., 2003a,b) and acoustical communication (Lindauer & Kerr, 1958; Esch *et al*., 1965; Nieh & Roubik, 1998; Nieh *et al*., 2003b; Nieh, 2004). Species vary considerably in their nest architecture, which ranges in design from brood cells arranged in horizontal combs or clusters, constructed within crevices in trees or in the ground (Wille & Michener, 1973; Roubik, 2006), and occasionally within the active colonies of other social insects (e.g. Schwarz, 1948; Rasmussen, 2004). The substantial elaboration of their nest entrance is generally species specific (Sakagami *et al*., 1990; Camargo & Pedro, 2003a; Franck *et al*., 2004). Furthermore, comparative studies of their oviposition rituals have identified high levels of diversity in reproductive behaviour (Sakagami & Zucchi, 1963; Sakagami, 1982; Zucchi *et al*., 1999; Drumond *et al*., 2000).

An important element in the diversity of stingless bees may be their relatively great age, as suggested in the fossil record and their worldwide distribution. The oldest known bee fossil to date is a meliponine (*Cretotrigona prisca*) from New Jersey amber that apparently dates to the Late Cretaceous (Michener & Grimaldi, 1988a, b; Grimaldi, 1999; Engel, 2000), although controversy surrounds the

Correspondence: Claus Rasmussen, Department of Entomology, 320 Morrill Hall, 505 S. Goodwin Ave., University of Illinois, Urbana, Illinois 61801, U.S.A. E-mail: clausr@life.uiuc.edu

estimate (Rasnitsyn & Michener, 1990; Rasnitsyn & Quicke, 2002). Meliponines are distributed throughout the tropical and subtropical parts of the Afrotropical, Australasian, Indo-Malayan and Neotropical Regions, exhibiting greatest abundance in New World Amazonian rain forest (Michener, 1979; Roubik, 1992; Camargo, 1994; Michener, 2000). Their age and distribution pattern suggest a Gondwanan vicariance origin for the group (Michener, 1979; Camargo & Wittmann, 1989), but dispersal models involving Laurasian/Australasian interchange have been proposed (Kerr & Maule, 1964; Wille, 1979). To date, no biogeographical hypothesis has been tested with phylogenetic evidence.

Evolutionary insight into the unusually diverse social behaviour and biogeographical history of the stingless bees requires a strongly supported phylogenetic framework, which until now has been lacking. In this paper, we provide the foundation for this framework. In the following, we describe the history of meliponine classificatory and phylogenetic research, with emphasis on the largest and most widespread of the genera, *Trigona sensu lato* (*s.l.*).

Classification and current phylogenetic status of Meliponini

Reduced wing venation, the presence of a penicillum (a brush of long stiff setae on the anterior apical outer margin of the hind tibia) and the reduction of the sting apparatus in females support the monophyly of Meliponini. The presence of a hind tibial corbicula (pollen basket) is shared with other corbiculate bees, including Euglossini (Michener, 1990). The relationship of meliponines to the other corbiculate tribes has been controversial, as the available morphological evidence argues for Apini as the sister group (Roig-Alsina & Michener, 1993; Schultz *et al*., 2001), whereas accumulating molecular evidence points to Bombini as most closely related (Cameron & Mardulyn, 2001, 2003; Lockhart & Cameron, 2001; Cameron, 2003; Thompson & Oldroyd, 2004). There may have been considerable extinction of corbiculate lineages (Engel, 2001), possibly further obscuring the morphological and behavioural transitions between the extant tribes.

The morphological diversity of meliponines has led some authors to recognize many supra-specific groups at the generic level (Table 1; e.g. Moure, 1961, 1971; Silveira *et al*., 2002; Camargo & Pedro, 2003b). Others have proposed a simpler classification to avoid a profusion of formal names (Table 1; e.g. Wille & Michener, 1973; Michener, 2000). For instance, Wille (1983) concluded that numerous names would be meaningful only for a minority of entomologists, whereas Sakagami (1982) found it convenient to use the multiplicity of names in his review of meliponine biology. He suggested that most, if not all, of the supra-specific groups are natural and exhibit clear behavioural differences. Here, we use Moure's (1971) proposed generic system to fully represent the known taxonomic diversity, and to allow us to test the monophyly and relationships between various groupings.

In the largest meliponine genus, *Trigona s.l.* (Table 1), workers share a morphological synapomorphy in which the keirotrichia (a dense field of minute, blunt setae) is restricted to a median longitudinal band on the inner hind tibia, and, in cross-section, the hind tibia forms a broad, raised median ridge (Michener, 1990). This has been utilized for placing more than 120 species into ten subgenera (Michener, 2000) from the Indo-Malayan/Australasian and Neotropical Regions. The first cladistic phylogeny of Meliponini by Michener (1990), based on an analysis of seventeen morphological characters, recovered *Melipona* as sister group to the remaining taxa, whereas *Trigona s.l.* encompassed species from both the Neotropical and Indo-Malayan/Australasian Regions. All Afrotropical taxa, except *Hypotrigona*, formed a single derived clade. Camargo & Pedro (1992a, 1992b) reassessed Michener's characters and proposed an alternative classification in which *Melipona* fell within a derived Neotropical clade. The *Trigona s.l.* clade included Neotropical and Indo-Malayan/Australasian taxa, as in Michener (1990), but, by contrast, all Afrotropical taxa appeared as a sister clade to the other meliponines.

Both phylogenies are poorly resolved and lack confidence estimates of the phylogenetic results. Other morphology-based phylogenies of Meliponini involve lower level studies of taxa within and between various Neotropical genera (Camargo, 1996; Roubik *et al*., 1997; Camargo & Pedro, 2003a, b, 2004; Pedro & Camargo, 2003; Camargo & Roubik, 2005). Phenetic analyses have been performed on a limited number of taxa (Pisani *et al*., 1969; Cunha, 1973, 1991, 1994). A molecular investigation of thirty-four species representing twenty-two genera, including three Indo-Malayan/Australasian and three Afrotropical genera, was undertaken by Costa *et al*. (2003), based on a 320–421-bp fragment of 16S rRNA. On the basis of their limited character and taxonomic sampling, they found that the Indo-Malayan/Australasian *Trigona* were related to Afrotropical taxa, whereas *Trigona sensu stricto* (*s.s.*) was a derived Neotropical clade. However, the maximum parsimony (MP) bootstrap values (BVs) for most relationships between genera were below 50%.

The knowledge of meliponine relationships is obscured by the scarcity of good morphological synapomorphies to reveal a consistent phylogenetic pattern (Michener, 1990; Camargo & Pedro, 1992a); furthermore, there remains a need to address the relationships of the Old World Meliponini and to test the monophyly of *Trigona s.l.* with a larger dataset.

In this paper, we report the results of a phylogenetic analysis of the inter- and infrageneric relationships of Old World meliponines (i.e. Afrotropical, Indo-Malayan and Australasian; *sensu* Olson *et al*., 2001) with a nearly complete sampling of genera (*sensu* Moure, 1971). We also examine the overall monophyly of *Trigona s.l.* with additional taxon sampling from the Neotropical Region. DNA sequences for these taxa were collected from four genes [mitochondrial 16S rRNA, nuclear long-wavelength rhodopsin copy 1 (opsin), elongation factor-1α copy F2 (EF-1α) and arginine kinase (ArgK)]. For comparison of

Table 1. The two classificatory systems of stingless bees (Meliponini). First column according to Michener (2000) and second column according to Moure (1951, 1961, 1971), including the authority of each generic name. Our study follows Moure in the use of names, except for *Melipona*, where we refer to *Eomelipona*, *Melikerria*, *Melipona s.s.* and *Michmelia* as subgenera of *Melipona*. *Paratrigonoides* (Neotropical) was described recently and is not included (Camargo & Roubik, 2005). Regional distributions of the taxa are Afrotropical (AT), Neotropical (NE), Indo-Malayan (IM) and Australasian (AA). Olson *et al.* (2001) considered Java, Bali, Borneo and the Philippines as the south-easternmost parts of the Indo-Malayan region, whereas Sulawesi was assigned to the Australasian region. Indo-Malayan taxa found in the Australasian Region only through their presence on Sulawesi (*Geniotrigona*, *Lepidotrigona*) are marked with an asterisk.

Genus *sensu* Michener	Genus *sensu* Moure	Distribution
Austroplebeia	*Austroplebeia* Moure	AA
Cephalotrigona	*Cephalotrigona* Schwarz	NE
Cleptotrigona	*Cleptotrigona* Moure	AT
Dactylurina	*Dactylurina* Cockerell	AT
Hypotrigona	*Hypotrigona* Cockerell	AT
Lestrimelitta	*Lestrimelitta* Friese	NE
Liotrigona	*Liotrigona* Moure	AT
Lisotrigona	*Lisotrigona* Moure	IM
Melipona	*Eomelipona* Moure	NE
Melipona	*Melikerria* Moure	NE
Melipona	*Melipona* Illger	NE
Melipona	*Michmelia* Moure	NE
Meliponula (*Meliplebeia*)	*Apotrigona* Moure	AT
M. (*Axestotrigona*)	*Axestotrigona* Moure	AT
M. (*Meliplebeia*)	*Meliplebeia* Moure	AT
M. (*Meliponula*)	*Meliponula* Cockerell	AT
M. (*Meliplebeia*)	*Plebeiella* Moure	AT
Meliwillea	*Meliwillea* Roubik, Segura & Camargo	NE
Nannotrigona	*Nannotrigona* Cockerell	NE
Nogueirapis	*Nogueirapis* Moure	NE
Oxytrigona	*Oxytrigona* Cockerell	NE
Paratrigona	*Aparatrigona* Moure	NE
Paratrigona	*Paratrigona* Schwarz	NE
Pariotrigona	*Pariotrigona* Moure	IM
Partamona (*Parapartamona*)	*Parapartamona* Schwarz	NE
P. (*Partamona*)	*Partamona* Schwarz	NE
Plebeia (*Plebeia*)	*Friesella* Moure	NE
Plebeia (*Plebeia*)	*Mourella* Schwarz	NE
Plebeia (*Plebeia*)	*Plebeia* Schwarz	NE
P. (*Scaura*)	*Scaura* Schwarz	NE
P. (*Schwarziana*)	*Schwarziana* Moure	NE
P. (*Scaura*)	*Schwarzula* Moure	NE
Plebeina	*Plebeina* Moure	AT
Scaptotrigona	*Sakagamilla* Moure	NE
Scaptotrigona	*Scaptotrigona* Moure	NE
Trichotrigona	*Trichotrigona* Camargo & Moure	NE
Trigona sensu lato		
Trigona (*Tetragona*)	*Camargoia* Moure	NE
T. (*Duckeola*)	*Duckeola* Moure	NE
T. (*Frieseomelitta*)	*Frieseomelitta* Ihering	NE
T. (*Heterotrigona*)	*Geniotrigona* Moure	IM*
T. (*Geotrigona*)	*Geotrigona* Moure	NE
T. (*Heterotrigona*)	*Heterotrigona* Schwarz	IM
T. (*Homotrigona*)	*Homotrigona* Moure	IM
T. (*Lepidotrigona*)	*Lepidotrigona* Schwarz	IM*
T. (*Heterotrigona*)	*Lophotrigona* Moure	IM
T. (*Heterotrigona*)	*Odontotrigona* Moure	IM
T. (*Papuatrigona*)	*Papuatrigona* Michener & Sakagami	AA
T. (*Heterotrigona*)	*Platytrigona* Moure	IM/AA
T. (*Tetragona*)	*Ptilotrigona* Moure	NE
T. (*Heterotrigona*)	*Sundatrigona* Inoue & Sakagami	IM
T. (*Tetragona*)	*Tetragona* Lepeletier & Serville	NE
T. (*Heterotrigona*)	*Tetragonilla* Moure	IM
T. (*Tetragonisca*)	*Tetragonisca* Moure	NE
T. (*Heterotrigona*)	*Tetragonula* Moure	IM/AA
T. (*Heterotrigona*)	*Tetrigona* Moure	IM
T. (*Trigona*)	*Trigona* Jurine	NE
Trigonisca	*Celetrigona* Moure	NE
Trigonisca	*Dolichotrigona* Moure	NE
Trigonisca	*Leurotrigona* Moure	NE
Trigonisca	*Trigonisca* Moure	NE

the results from Bayesian Markov chain Monte Carlo (BMCMC) analysis, both maximum likelihood (ML) and MP inference methods were included.

Materials and methods

Taxon sampling

Seventy-nine taxa comprise the ingroup (Table S1, Supplementary material), including all proposed Old World genera and subgenera (Moure, 1971), except *Papuatrigona* (monotypic, New Guinea), *Pariotrigona* (monotypic, Thailand, Malaysia, Borneo, Sumatra) and *Cleptotrigona* (monotypic, sub-Saharan Africa) (Michener, 1990, 2000, 2001; Sakagami *et al.*, 1990; Eardley, 2004). Neotropical taxa include fifteen exemplars representing thirteen taxonomically diverse genera, including several *Trigona s.s.* and distantly related groups. Six taxa from the other corbiculate tribes were selected as outgroups. Table S1 (Supplementary material) provides a list of the species, their collection localities, voucher and GenBank accession numbers. If colour polymorphism or disjunct distribution suggested

more than one biological species in the available material, multiple individuals were included to assess the degree of sequence divergence (i.e. *Tetragonula fuscobalteata*, *T. clypearis*, *T. geissleri*, *T. sapiens*, *Heterotrigona itama*, *Lepidotrigona terminata*, *Geniotrigona thoracica*, *Axestotrigona ferruginea* and *Austroplebeia symei*).

Gene selection

We sequenced fragments of four independently evolving genes found to be informative for the phylogenetic analysis of bees at several levels of relationship: mitochondrial 16S for closely related species (Cameron & Williams, 2003; Cameron *et al*., 2006; Hines *et al*., 2006) and, for resolution at higher levels, nuclear opsin copy 1 (Mardulyn & Cameron, 1999; Ascher *et al*., 2001; Cameron & Mardulyn, 2003; Cameron & Williams, 2003; Lin & Danforth, 2004; Ortiz-Rivas *et al*., 2004; Spaethe & Briscoe, 2004), ArgK (Kawakita *et al*., 2003, 2004; Hines *et al*., 2006) and EF-1α copy F2 (Cho *et al*., 1995; Danforth & Ji, 1998; Danforth *et al*., 1999).

DNA extraction, amplification and sequencing

Bees were collected into 95% ethanol in the field and stored at 4 °C in the laboratory. DNA was extracted from thoracic muscle of single individuals using the Dneasy® tissue extraction kit (QIAGEN Inc., Valencia, California), resuspended into 100–180 μl buffer TE, depending on the amount of available tissue. A limited number of taxa were extracted using phenol–chloroform with ethanol precipitation. For small specimens, the entire insect except head and wings was used. Voucher remains of all sampled taxa are retained at the Illinois Natural History Survey, Champaign, Illinois, U.S.A.

Polymerase chain reaction (PCR) amplification and DNA sequencing were performed with primers reported from the literature for 16S (Cameron *et al*., 1992: 874-16S1R; Dowton & Austin, 1994: 16SWb), opsin (Mardulyn & Cameron, 1999), EF-1α (Hines *et al*., 2006) and ArgK (Kawakita *et al*., 2003: Kawakita 2F/2R). Some meliponine taxa did not amplify successfully with the reverse opsin primer, but did so with a degenerate oligo (5′-CACTCCGYACTRGTATTYTGAT-3′) that terminated upstream of the second intron.

Double-stranded PCR products were amplified with an Eppendorf Mastercycler gradient (Eppendorf AG, Hamburg, Germany) PCR machine using an initial denaturation step for 2–5 min at 94–95 °C, followed by thirty-five cycles of denaturation (60 s at 94–95 °C), annealing and elongation (48 °C/68 °C for 16S, 49 °C/65 °C for opsin, 53–55 °C/72 °C for EF-1α, 50 °C/65 °C for ArgK). A final extension was run for 4–5 min at 65–72 °C. PCR products were purified using the QIAquick® spin kit (QIAGEN) according to the manufacturer's protocol. DNA sequencing was carried out with PCR primers using the BigDye® terminator kit version 3.1 (Applied Biosystems, Foster City, California). Sequence products were run on an ABI 3730XL automated sequencer (W. M. Keck Center for Comparative and Functional Genomics, University of Illinois, Champaign, Illinois, U.S.A.). Both strands were sequenced for all taxa. Sequences of ArgK are missing for five taxa and of EF-1α for one taxon (Table S1, Supplementary material). Sequences are deposited in GenBank under the accession numbers given in Table S1 (Supplementary material).

Sequence alignment

DNA sequences were edited and aligned in BioEdit version 7.0.0 (Hall, 1999) with costs of 20 for opening and 0.1 for extension. Computer alignments were adjusted by hand to optimize positional homology, in particular within introns and variable regions. Intron, variable and gap regions were alignable and included in the analyses because they yield phylogenetically useful characters (Cameron & Williams, 2003; Kawakita *et al*., 2003; Hines *et al*., 2006).

Uncorrected pairwise sequence divergences (*p* distances) were calculated in paup* version 4.0b10 (Swofford, 2002). These values were used to estimate divergence between all sequences and to compare mean divergence between sequences for each gene. To test for stationarity, base composition statistics were evaluated with a chi-squared (χ^2) test for homogeneity in paup*.

Phylogenetic methods

Bayesian analyses. Bayesian analysis with an MCMC search strategy was implemented in MrBayes version 3.1.2 (Ronquist & Huelsenbeck, 2003). Nuclear genes were partitioned into exons and introns to allow for variable evolutionary rates between gene regions (Huelsenbeck & Crandall, 1997). The most appropriate substitution model was determined for each gene partition on the basis of the Akaike information criterion (AIC) in Modeltest version 3.7 (Posada & Crandall, 1998). Each model is listed in Table 2. Three replicate independent BMCMC analyses (four chains, mixed models, flat priors, trees sampled every 1000 generations) were run for each gene fragment (two million generations) and for a combined genes dataset (eight million generations). Log likelihood plots of trees from the Markov chain samples were examined in Tracer version 1.3 (Rambaut & Drummond, 2006) to determine convergence to a stable log likelihood value. All trees estimated prior to convergence (burn-in) (Huelsenbeck & Ronquist, 2001) were discarded. Likelihood traces between replicate runs were compared for convergence to similar log likelihood values. If replicate runs converged, all trees after burn-in were combined to create a single consensus tree. BMCMC posterior probability (PP) values represent the proportion of MCMC samples that contain a particular node.

ML non-parametric bootstrapping (Felsenstein, 1985), applying the GTR + I + G model, was performed for

Table 2. Descriptive results for each gene and gene partition with relevant model selected for each partition. Mean uncorrected distance within the largest genus, *Tetragonula*, was 3.41% (0.16–5.96%).

Gene partition	Number of sites	Parsimony informative sites	Mean (range) uncorrected distance (ingroup, IG) (%)	Mean (range) uncorrected distance (all taxa) (%)	A/T (IG) (%)	Model (IG)	Gamma shape parameter (IG)	N_{st} (IG)	Base composition homogeneity test (χ^2; d.f. = 252/ *P* value)
All genes	2814	895	6.2 (0–11.1)	7.7 (0–22.7)	60.8	TVM + I + G	0.4985	6	225.48/0.884
16S	574	208	10.1 (0–15.9)	11.3 (0–26.6)	75.9	K81 + I + G	0.3978	6	231.63/0.817
Opsin	580	201	4.7 (0–11.0)	6.4 (0–22.7)	57.1	–	–	–	40.43/1.000
Op exon	459	140	3.7 (0–9.2)	5.3 (0–20.7)	54.8	GTR + I + G	0.8897	6	24.83/1.000
Op intron	121	61	10.1 (0–26.5)	13.0 (0–50.8)	69.6	HKY + G	2.5600	2	69.06/1.000
ArgK	843	259	6.2 (0–13.2)	8.0 (0–29.1)	51.3	–	–	–	204.12/0.988
Arg exon	551	167	4.8 (0–9.3)	6.3 (0–19.4)	49.3	TVM + I + G	0.5351	6	69.19/1.000
Arg intron	292	92	21.9 (0–63.2)	27.6 (0–76.6)	69.9	HKY + G	4.7863	2	187.07/0.999
EF-1α	817	227	4.5 (0–8.1)	5.8 (0–17.3)	60.0	–	–	–	25.14/1.000
EF exon	738	195	4.3 (0–7.7)	5.4 (0–16.2)	59.3	TrN + I + G	1.0086	6	20.29/1.000
EF intron	79	32	9.4 (0–28.0)	14.1 (0–62.9)	74.7	TrN + I	equal	6	88.04/1.000

16S, mitochondrial 16S rRNA; opsin, nuclear long-wavelength rhodopsin copy 1; EF-1α, elongation factor-1α; ArgK, arginine kinase; N_{st}, number of substitution types.

comparison of BVs with PPs. ML BVs were estimated in PHYML version 2.4.4 (Guindon & Gascuel, 2003) (200 replicates, GTR model, p-invar = 0.49, gamma distribution = 0.50) based on an ML starting tree obtained in PAUP* [100 replicates, tree bisection–reconnection (TBR) branch swapping, retaining 500 trees per replicate]. When all partitions were combined, MODELTEST suggested the GTR + I + G model (log likelihood, – 19 655.6) second to the TVM + I + G model (log likelihood, – 19 656.3). Only the GTR model was supported by PHYML and therefore used in the analysis.

Parsimony analyses. Both MP (heuristic search, 1000 random additions, TBR branch swapping, all characters of equal weight) and bootstrap (heuristic search, 500 replicates, ten random additions per replicate, retaining 200 trees per replicate) analyses were performed in PAUP*. Bremer support values (Bremer, 1994) were calculated in TREEROT version 2 (Sorensen, 1999).

Nodes that received ≥ 0.95 PP or $\geq 70\%$ BV were considered to be well supported. The Incongruence Length Difference (ILD) (Farris *et al*., 1995; Sanderson & Shaffer, 2002), implemented in PAUP*, was used to assess data compatibility for each pair of partitions (heuristic search, 100 replicates, ten random taxon additions per replicate, TBR branch swapping, retaining 500 trees per replicate).

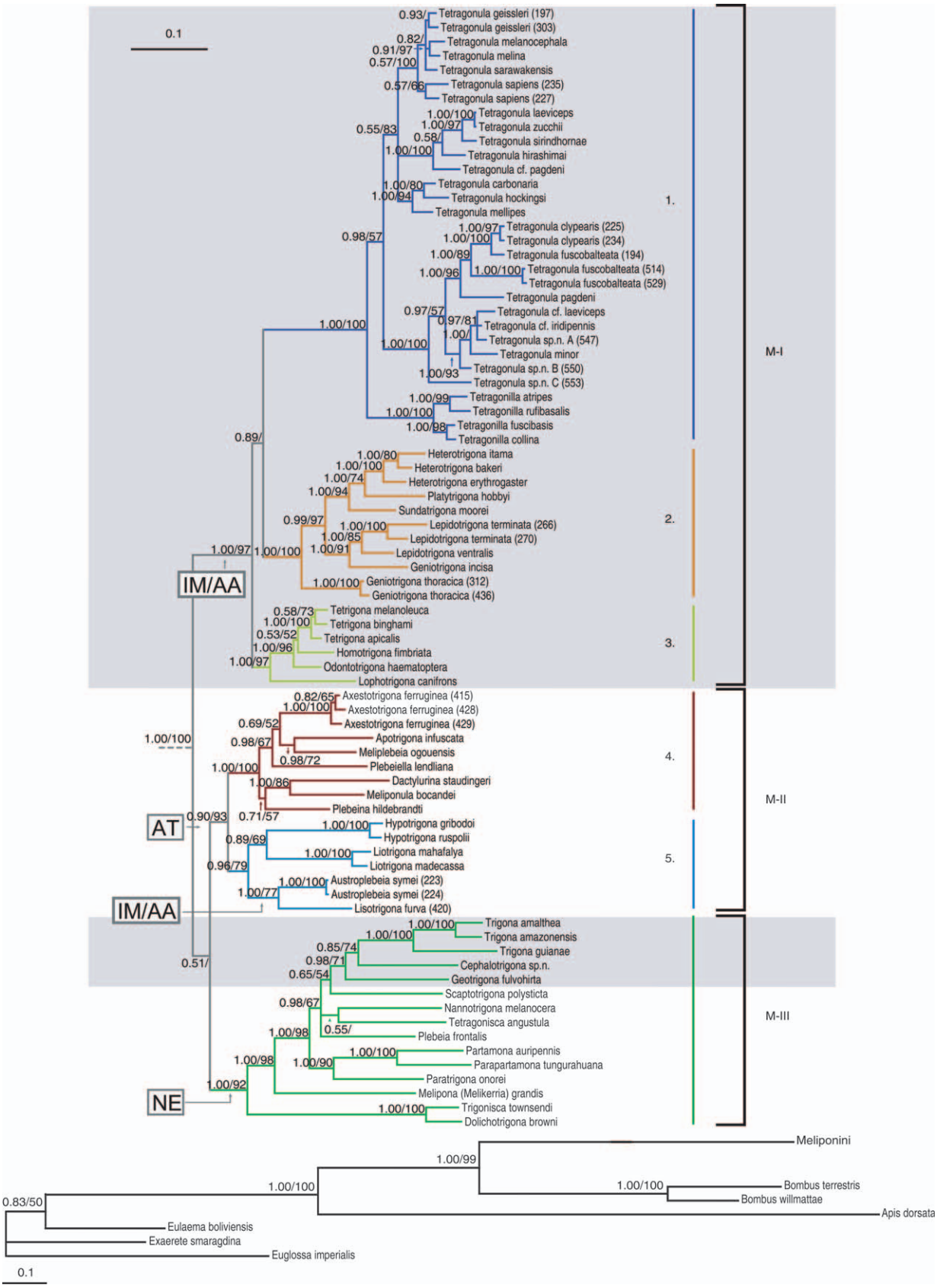
Results

Data characteristics

The combined dataset consisted of 2814 aligned nucleotides for the four gene fragments: 574 bp of 16S, 580 bp of opsin, 843 bp of ArgK and 817 bp of EF-1α. The total numbers of parsimony informative sites for each partition and gene are listed in Table 2. The overall base composition was AT biased (60.9%; Table 2) and the within-gene AT bias (51.3% for ArgK, 57.1% for opsin, 60.0% for EF-1α, 75.9% for 16S) was comparable with that reported for other Hymenoptera (Whitfield & Cameron, 1998; Ascher *et al*., 2001; Cameron & Williams, 2003; Hines *et al*., 2006). Nuclear introns showed higher AT bias (69.6–74.7%) than exons, which had no or only slight bias (49.3–59.3%). Mean uncorrected pairwise distances for the ingroup alone and for the ingroup plus outgroup are given for each partition in Table 2. There are large differences in sequence divergence between exon and intron regions of each nuclear gene (Table 2: 3.7% vs. 10.1%, respectively, for opsin; 4.8% vs. 21.9% for ArgK; 4.3% vs. 9.4% for EF-1α). Introns varied in length between the ingroup taxa and together contained 27% of the informative sites for nuclear genes.

The ILD tests indicated no significant ($P > 0.1$) incongruence between the partitions (data not shown), except for three values at $P = 0.01$ (opsin exon/ArgK intron; opsin exon/EF-1α exon; ArgK intron/EF-1α exon). Because incongruence was not strongly supported and problems

Fig. 1. Meliponini phylogeny estimated from Bayesian analysis of four gene fragments [mitochondrial 16S rRNA, nuclear long-wavelength rhodopsin copy 1 (opsin), elongation factor-1α copy F2 (EF-1α) and arginine kinase (ArgK)]. Black brackets indicate the three major (M) clades recovered (M-I, M-II, M-III). Internal clades are further represented by corresponding vertical numbered and/or coloured lines and branches. These lineages represent groupings of the Indo-Malayan/Australasian Regions (M-I, group 1–3), Afrotropical Region (M-II, group 4), Afrotropical/Indo-Malayan/Australasian Regions of 'minute' Meliponini (M-II, group 5) and Neotropical Region (M-III). Regions are further indicated as Afrotropical (AT), Australasian (AA), Indo-Malayan (IM) and Neotropical (NE). The shaded areas represent taxa comprising the conventional view of '*Trigona*' *s.l.* Values above the branches are BMCMC posterior probabilities (PP)/maximum likelihood bootstrap values (ML BV). PP values without accompanying ML BV values reflect low support (ML BV < 50%) or ML polytomies, except for *Tetragonula minor* (ML BV = 64%) and *T.* **sp.n.** B, whose positions were reversed in the ML analysis.



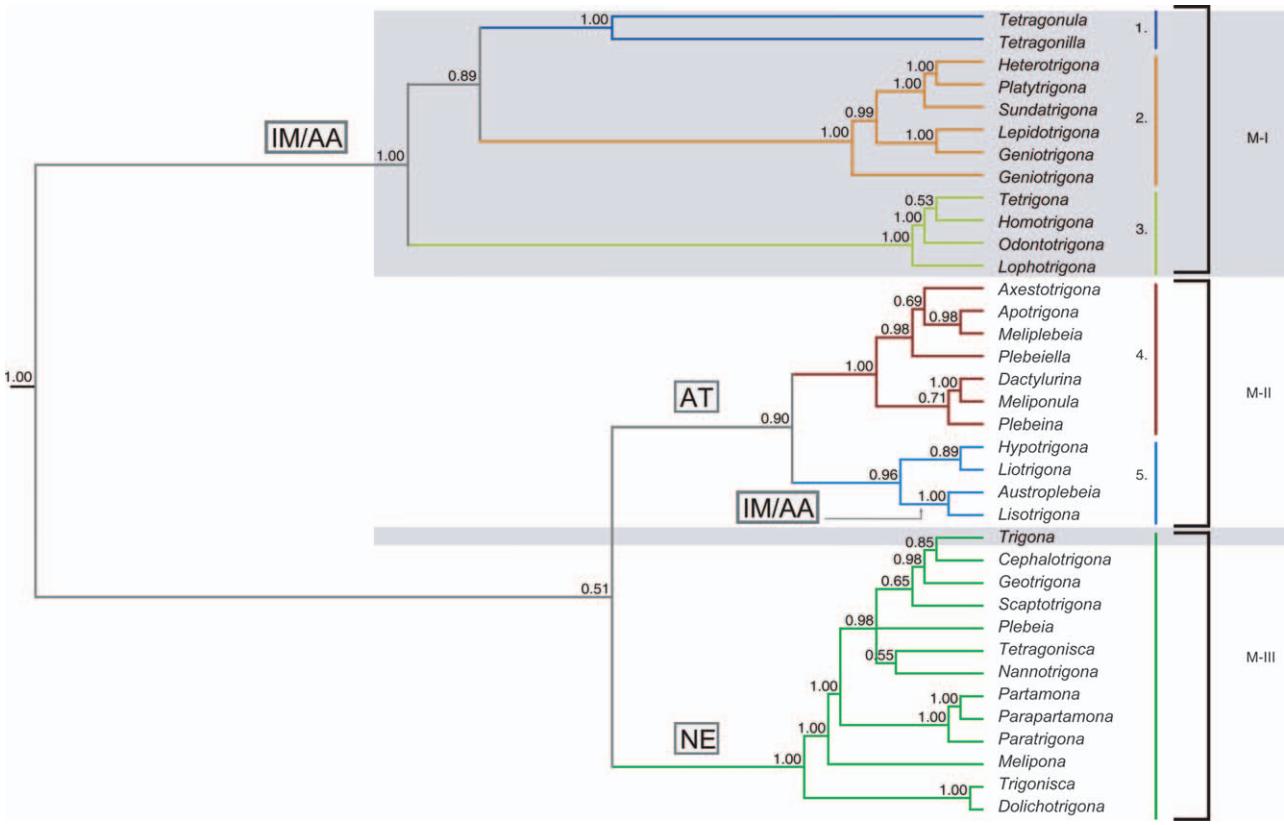


Fig. 2. Summary tree of Fig. 1, indicating the meliponine relationships estimated from Bayesian analysis. Values above the branches are posterior probabilities. Brackets and colour coding are as described in Fig. 1.

associated with the test were reported by Dowton & Austin (2002), all four datasets were combined in analyses.

Relationships

Analyses of individual gene fragments resulted in relatively well-resolved phylogenies (not shown). Three of the four genes divided the taxa into the same three major clades, distinguishing the Indo-Malayan/Australasian, Afrotropical and Neotropical taxa discussed further below: opsin (PP 1.00/0.55/0.98), EF-1α (PP 0.91/0.85/–) and 16S (PP 0.85/0.54/1.00). The missing EF-1α value is due to a polytomy. ArgK recovered supra-specific groups, but provided no support for the three major clades. Both 16S and ArgK provided good resolution within genera, near the tips of the tree.

The BMCMC analysis of the combined gene sequences (Fig. 1; summary tree in Fig. 2) provides strong support for the same three principal clades found within the individual gene analyses: an Indo-Malayan/Australasian major clade [major clade I (hereafter M-I); PP = 1.00, ML BV = 97%], an Afrotropical major clade (M-II; PP = 0.90, ML BV = 93%) and a Neotropical major clade (M-III; PP = 1.00, ML BV = 92%). The only taxa that contradict these biogeographically defined clades are *Lisotrigona* (Indo-Malayan) and *Austroplebeia* (Australasia), both of which fall within the Afrotropical clade.

MP provides strong support for an M-I clade (Fig. 3; MP BV = 100%), no support for M-II (MP BV < 50%) and weak support for M-III (MP BV = 65%). Of the forty-two nodes that correspond between the ML and MP trees, the ML BVs were on average 7% higher than the MP BVs. Highly supported nodes (BV = 95%) were consistently well supported by both optimality criteria. Only two well-supported nodes under ML (92%/86%) received poor support under MP (65%/62%).

Although the entire M-I clade comprises all of the Indo-Malayan/Australasian *Trigona*, the Neotropical *Trigona* occur as a derived group within the M-III clade (PP = 0.98, ML BV = 71%). Thus, *Trigona s.l.* is not monophyletic.

The M-I clade comprises three well-supported groups: *Tetragonula–Tetragonilla*, *Heterotrigona–Geniotrigona* and *Tetrigona–Lophotrigona* (Figs 1 and 2, groups 1–3; PP = 1.00, ML BV = 97–100%). The same three groups are supported under MP (Fig. 3). Within group 2, *Geniotrigona* is not monophyletic because *G. thoracica* is sister to the remaining taxa (PP = 1.00, ML BV = 97%), whereas *G. incisa* is sister to a derived *Lepidotrigona* clade (PP = 1.00, ML BV = 91%).

The well-supported Afrotropical clade comprises two lineages: *Axestotrigona–Plebeina* (group 4; PP = 1.00, ML BV = 100%) and *Hypotrigona–Lisotrigona* (group 5; PP = 0.96, ML BV = 79%), the latter including Indo-Malayan/

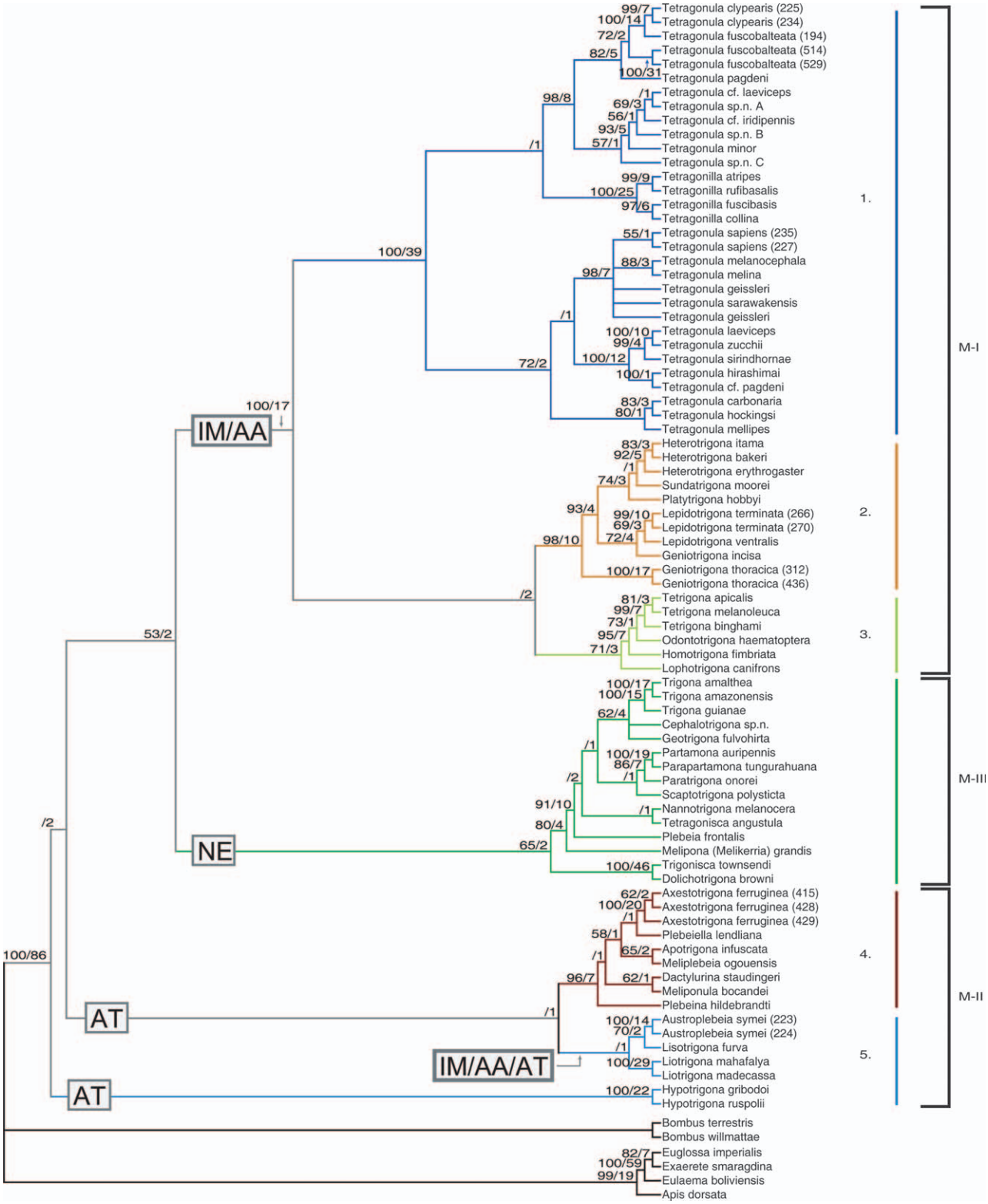


Fig. 3. Meliponini phylogeny (strict consensus of 12 trees) estimated from maximum parsimony (MP) analysis of four gene fragments [Tree length, TL = 4.293, consistency index (CI) = 0.36, retention index (RI) = 0.71]. Values above the branches are MP bootstrap values ≥ 50/ Bremer support indices. Brackets and colour coding are as described in Fig. 1.

Australasian *Lisotrigona* and *Austroplebeia*. MP consistently recovered group 4 with high support (MP BV = 96%), but group 5 was poorly supported (MP BV < 50%), with *Hypotrigona* falling outside as a distinct lineage (MP BV < 50%).

In addition to the polyphyletic division of *Trigona s.l.* within the Neotropical clade, the genus *Melipona s.l.* was recovered in a relatively basal position as sister to the majority of New World taxa (PP = 1.00, ML BV = 98%).

Interspecific nucleotide differences

Of the nine polymorphic species included in the analyses, all were recovered as sister taxa, except for *T. fuscobalteata* (Fig. 1), in which two specimens from Borneo (only 4 bp difference across all genes) were 7.4% different (16S) from a specimen from Sulawesi (68 bp and 70 bp difference for all genes, respectively, for the vouchers 514 and 529). Smaller differences were found in the other taxa: *T. sapiens* 2.2% different for 16S (30 bp/all genes), *Heterotrigona itama* and *H. bakeri* 3.8% for 16S (30 bp/all genes) and *Lepidotrigona terminata* 4.4% for 16S (43 bp/all genes). *T. clypearis*, *T. geissleri*, *G. thoracica*, *Axestotrigona ferruginea* and *Austroplebeia symei* were 0–16 bp different across all genes (< 2.7% for 16S).

Discussion

Non-monophyly of Trigona

With comprehensive taxon sampling of nearly all the Old World meliponine genera (*sensu* Moure, 1971) and exemplars of a broad diversity of Neotropical taxa, we have shown that the conventional *Trigona s.l.* is not monophyletic. Rather, it divides into a Neotropical clade and a distantly related Indo-Malayan/Australasian clade. Jurine (1807) erected the genus *Trigona* for three nominal Neotropical species (*amalthea*, *favosa*, *ruficrus*) without an indication of which one was the name-bearing type. Subsequently Latreille (1810) designated *Trigona amalthea* as the type species for the genus. To recognize monophyletic groups and to uphold the nomenclatural principle of priority, we recommend that the generic name *Trigona* be applied only to the Neotropical taxa and that the use of the name *Trigona* for the Indo-Malayan/Australasian taxa be discontinued. With regard to the question of whether to recognize higher groupings as genera (*sensu* Moure, 1971) or as subgenera (*sensu* Michener, 2000), this is an arbitrary decision about rank. The critical issue is that recognized groups are monophyletic. This phylogeny helps to reinforce these groups. A detailed consideration of overall meliponine classification will be published elsewhere.

Relationships between Indo-Malayan/Australasian genera

The single clade of Indo-Malayan/Australasian *Trigona*-like taxa (M-I) is also supported by a morphological synapomorphy of the hind leg (Camargo & Pedro, 1992a, b), namely the polished surface of the posterobasal part of the hind basitarsus is delimited anteriorly by a low ridge bearing a row of setae. All of the M-I taxa were included by Michener (1990, 2000) in the four *Trigona* subgenera *Heterotrigona* (thirty-six species), *Lepidotrigona* (four species), *Homotrigona* (monotypic) and *Papuatrigona* (monotypic). He recognized their close affinity as a group, but presented no hypothesis of their relationships. His subgenus *Heterotrigona* was treated as a diverse group comprising all M-I taxa, except *Lepidotrigona*, *Homotrigona* and *Papuatrigona*. This classification scheme is contradicted by our findings that *Heterotrigona* (*sensu* Michener, 2000) is polyphyletic.

Geniotrigona is polyphyletic due to placement of *G. incisa* within the *Lepidotrigona* clade: correction of the polyphyletic *Geniotrigona* could be made by expanding the concept of *Lepidotrigona* to include *G. incisa*. However, others have argued that *Lepidotrigona* is morphologically and behaviourally distinct based on the unique plumose or scalelike hairs along the margin of the mesoscutum (Schwarz, 1939) and its oviposition rituals (cf. Yamane *et al.*, 1995). Therefore, it is appealing to retain *Lepidotrigona* as a well-defined group by proposing a new suprageneric name for *G. incisa*.

Tetragonula is the single largest and most widespread genus in the Indo-Malayan/Australasian Regions, reported from India to the Solomon and Caroline Islands. Sakagami (1978) revised only species from continental Asia and suggested several species groups (Sakagami *et al.*, 1990). Evidently, the genus needs taxonomic revision given that six of our twenty-two sampled species are either newly discovered and undescribed or genetically distinct (*T. fuscobalteata*). We propose the construction of six species groups based on the high support values of taxa sampled: *geissleri–sapiens*; *laeviceps*–cf. *pagdeni*; *carbonaria–mellipes*; *clypearis–pagdeni*; cf. *laeviceps*–sp.n. B; sp.n. C.

Relationships between the Afrotropical genera

The Afrotropical meliponine fauna is less diverse than the Neotropical or Indo-Malayan/Australasian faunas, based on its relatively fewer species (nineteen species; Eardley, 2004) and genera (ten genera; Moure, 1961), and its low abundance in most parts of Africa (although see Darchen, 1972; Kajobe & Roubik, 2006). The taxa (excluding *Hypotrigona*) are united morphologically by the worker gonostyli, which are enlarged, apically diverged and covered with micropilosity (Camargo & Pedro, 1992a, b). Bayesian MCMC and ML give reasonably good support for a placement of *Hypotrigona* within the Afrotropical clade.

A reduced rastellum and the presence of a well-developed posterior parapenicillum on the worker hind tibia have been considered morphological synapomorphies linking *Axestotrigona*, *Apotrigona*, *Meliplebeia*, *Meliponula* and *Plebeiella* (Wille, 1979; Michener, 1990; Camargo & Pedro, 1992b).

Our results support this grouping plus *Dactylurina* and *Plebeina*. *Plebeina* possesses a well-developed rastellum but an undeveloped posterior parapenicillum, like that found in Neotropical *Plebeia s.s.* In *Dactylurina*, the rastellum is weakly developed (Camargo & Pedro, 1992b). *Dactylurina* has been proposed (Engel, 2000) as the closest relative to the Late Cretaceous age *Cretotrigona*, but the phylogenetic position of the fossil remains tentative.

Placement of the 'minute' Meliponini

Genera of minute or small extant taxa [*Austroplebeia*, *Cleptotrigona* (not included here), *Hypotrigona*, *Liotrigona*, *Lisotrigona*, *Pariotrigona* (not included here), *Trigonisca s.l.*] are found throughout the natural range of the stingless bees, perhaps as an adaptation to the occupancy of small nest cavities (Michener, 1961, 2001). Their small size has led to convergent reduction of at least wing venation (Moure, 1961; Michener, 1990, 2001), making phylogenetic placement based on morphology difficult as a result of a lack of good synapomorphies (Michener, 1990, 2001). *Austroplebeia*, for example, has been ascribed to Afrotropical (Michener, 1990) and Neotropical (Camargo & Pedro, 1992a) taxa. Our results show that the minute *Trigonisca s.l.* belongs to the Neotropical clade (M-III), and that *Liotrigona*, *Hypotrigona*, *Lisotrigona* and *Austroplebeia* comprise a monophyletic lineage (group 5) of minute bees within the Afrotropical clade. The minute *Cleptotrigona* and *Pariotrigona* remain to be sampled, but otherwise there appears to have been a convergent reduction in size and wing venation between Neotropical *Trigonisca s.l.* and the *Hypotrigona–Lisotrigona* group.

Other generic placements

The Neotropical clade (M-III) provides insights into the relationships of some of its groups (thirteen of thirty-five supra-specific groups sampled here), and additional taxa are being sequenced to elucidate the relationships of this taxonomically and biologically diverse clade. Of particular interest with respect to these results is the placement of *Melipona*, which, by contrast with Michener (1990), is not sister to the remaining meliponines, but instead falls within the Neotropical clade with strong support.

Polymorphic species

Nucleotide differences between multiple representatives of a species generally did not indicate the presence of polytypic taxa. However, a single *T. fuscobalteata* from Sulawesi (194) was not conspecific with morphologically similar taxa from west of the Wallace line (68–70 bp difference across genes). Similarly, *Lepidotrigona terminata* (east and west of the Wallace line) and *T. sapiens* (Australia and New Guinea) showed high sequence divergence (30–43 bp). Additional biological and morphological examination will probably support these island sister species splits.

Heterotrigona itama has been considered a single species from Thailand, Malaysia, Borneo, Sumatra and Java, with variable wing coloration ranging from dark fuscous to transparent (Schwarz, 1939). Our results suggest that at least two cryptic species may be present. These molecular data are consistent with field observations from Borneo (Sabah, Malaysia), which reveal distinct nest entrances between the two forms (C. Rasmussen, unpublished). Examination of the primary types of *H. itama* and its junior synonym, *H. bakeri*, in the National Museum of Natural History (Washington DC) indicates that the two forms sequenced correspond to *H. itama* and *H. bakeri* (C. Rasmussen, pers. obs.). *H. bakeri* (Cockerell, 1919) **stat.n.** is therefore distinct and corresponds to the fuscous morph.

Biogeographical considerations

Several Neotropical taxa (*Trigona s.l.*, *Plebeia s.l.* and *Trigonisca s.l.*) were considered to be close relatives of Old World taxa on the basis of morphology (e.g. Moure, 1950; Wille & Michener, 1973; Wille, 1979; Michener, 1990). These relationships have inspired, but also complicated, biogeographical hypotheses that can now be revisited with a strongly supported phylogeny. Kerr & Maule (1964) suggested that Meliponini originated and diversified in South America and then dispersed via the Nearctic and Palaearctic Regions during the Eocene to their current distribution. Wille (1979) proposed an out-of-Africa hypothesis in which Meliponini originated in Africa during the Late Cretaceous or early Tertiary and then dispersed via Europe during the Eocene and later to their current range. Considering *Plebeia s.l.* (Table 1: *Meliplebeia*, *Plebeiella*, *Plebeina*, *Austroplebeia* and Neotropical *Plebeia s.l.*) to be a single clade with taxa present in all geographical regions, but the Indo-Malayan Region, Camargo & Wittmann (1989) argued for a Gondwanan origin of the *Plebeia* lineage. Their explanation for the absence of *Plebeia* relatives in the Indo-Malayan Region was the existence of a land connection from the Patagonian shields to Antarctica, which could have allowed them to reach Australia via this land connection, without dispersal through the Indo-Malayan Region.

However, our results indicate that the *Plebeia* lineage is actually two distantly related clades, one Neotropical and the other Afrotropical/Indo-Malayan/Australasian, which refutes the Gondwanan origin of at least *Plebeia s.l.* All of group 5 (Fig. 2), with Australasian *Austroplebeia* and Indo-Malayan *Lisotrigona* included within an otherwise Afrotropical lineage, appears to have originated via dispersal from Africa. A similar dispersal scenario from Africa to Australasia has been suggested for *Braunsapis* (Apidae: Xylocopinae) (Fuller *et al.*, 2005), which originated in tropical Africa during the early Miocene, dispersed into Asia about 17 million years ago and arrived in Australia

during the late Miocene (Fuller *et al*., 2005). Neither the hypothesized South American origin of Meliponini (Kerr & Maule, 1964), nor the out-of-Africa hypothesis (Wille, 1979), can be tested here because of limited support for the basal relationships between the three major clades.

Seven species of *Tetragonula* found in Australia (Dollin *et al*., 1997; Franck *et al*., 2004) belong to three confirmed species groups (here named *geissleri–sapiens*, *carbonaria–mellipes* and *clypearis–pagdeni*), probably representing three distinct dispersal events into Australia from the Indo-Malayan Region (Franck *et al*., 2004). Although the *carbonaria–mellipes* species group is the predominant *Tetragonula* group in Australia, the other two groups are found widely outside Australia, and their establishment could have occurred more recently when periodic Pleistocene land bridges connected Australia via the Cape York Peninsula and New Guinea, the last of which was broken about 10 000 years ago (Dollin *et al*., 1997; Voris, 2000; Franck *et al*., 2004).

Implications for the classification of Meliponini and future directions

Our results strongly support the division of stingless bees into three major clades that correspond to the Indo-Malayan/Australasian, Afrotropical and Neotropical Regions. Biological studies of ecology, behaviour, nest architecture, etc. of the Old World taxa are greatly needed for comparison with these molecular results. With additional genes and a more complete taxon sampling from the Neotropical clade, we should be able to resolve the relationships between these three major clades. At that stage, a reclassification of the Meliponini would seem appropriate.

Supplementary material

The following material is available from http://www.blackwell-synergy.com. Table S1. Meliponine taxa included in this study, with voucher codes, collecting localities and GenBank accession numbers.

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