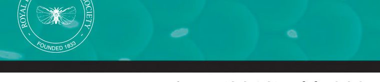

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The molecular phylogenetics of *Trachymyrmex* Forel ants and their fungal cultivars provide insights into the origin and coevolutionary history of 'higher-attine' ant agriculture

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Abstract. The fungus-growing ants and their fungal cultivars constitute a classic example of a mutualism that has led to complex coevolutionary dynamics spanning *c*. 55–65 Ma. Of the five agricultural systems practised by fungus-growing ants, higher-attine agriculture, of which leaf-cutter agriculture is a derived subset, remains poorly understood despite its relevance to ecosystem function and human agriculture across the Neotropics and parts of North America. Among the ants practising higher-attine agriculture, the genus *Trachymyrmex* Forel, as currently defined, shares most-recent common ancestors with both the leaf-cutter ants and the higher-attine genera *Sericomyrmex* Mayr and *Xerolitor* Sosa-Calvo *et al.* Although previous molecular-phylogenetic studies have suggested that *Trachymyrmex* is a paraphyletic grade, until now insufficient taxon sampling has prevented a full investigation of the evolutionary history of this group and limited the possibility of resolving its taxonomy. Here we describe the results of phylogenetic analyses of 38 *Trachymyrmex* species, including 27 of the 49 described species and at least 11 new species, using four nuclear markers, as well as phylogenetic analyses of the fungi cultivated by 23 species of *Trachymyrmex* using two markers. We generated new genetic data for 112 ants (402 new gene sequences) and 95 fungi (153 new gene sequences). Our results corroborate previous findings that *Trachymyrmex*, as currently defined, is paraphyletic. We propose recognizing two new genera, *Mycetomoellerius* **gen.n.** and *Paratrachymyrmex* **gen.n.**, and restricting the continued use of *Trachymyrmex* to the clade of nine largely North American species that contains the type species [*Trachymyrmex septentrionalis* (McCook)] and that is the sister group of the leaf-cutting ants. Our fungal cultivar phylogeny generally corroborates previously observed broad patterns of ant–fungus association, but it also reveals further violations of those patterns. Higher-attine fungi are divided into

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two groups: (i) the single species *Leucoagaricus gongylophorus* (Möller); and (ii) its sister clade, consisting of multiple species, recently referred to as *Leucoagaricus* Singer 'clade B'. Our phylogeny indicates that, although most non-leaf-cutting higher-attine ants typically cultivate species in clade B, some species cultivate *L. gongylophorus*, whereas still others cultivate fungi typically associated with lower-attine agriculture. This indicates that the attine agricultural systems, which are currently defined by associations between ants and fungi, are not entirely congruent with ant and fungal phylogenies. They may, however, be correlated with as yet poorly understood biological traits of the ants and/or of their microbiomes.

Introduction

The fungus-growing ('attine') ants (Formicidae: Myrmicinae: Attini: Attina) evolved *c.* 55–65 Ma in the tropical rainforests of South America (Wilson, 1971; Mueller *et al.*, 2001; Schultz & Brady, 2008; Branstetter *et al.*, 1975). The defining biological trait of this group is their obligate association with fungi cultivated inside their nests, which serve as the ants' primary food source (Möller, 1893; Weber, 1972; Hölldobler & Wilson, 1990). In return, the ants provide the fungi with protection, nourishment and dispersal. As such, the fungus-growing ants and their fungi constitute one of the best-studied examples of a mutualism between an insect and a microorganism (Boucher, 1988; Hölldobler & Wilson, 1990; Chapela *et al.*, 1994; Mueller *et al.*, 2005; Mehdiabadi & Schultz, 2010; Della Lucia, 2011; Hölldobler & Wilson, 2011).

Two hundred and forty-five species of fungus-growing ants have been described, which range across the New World from the U.S.A. to Argentina (Wheeler, 1907; Weber, 1972; Hölldobler & Wilson, 1990; Mayhé-Nunes & Jaffé, 1998; Fernández & Sendoya, 2004). Five distinct agricultural systems have been recognized. Each is broadly characterized by associations between phylogenetic groups (clades or grades) of ants and corresponding phylogenetic groups of fungi that are parasitized by phylogenetic groups of parasitic ascomycete fungi in the genus *Escovopsis*, as well as by the substrate upon which the fungi are cultivated (Weber, 1972; Gerardo *et al.*, 2004, 2006; Schultz & Brady, 2008; Mehdiabadi & Schultz, 2010; Meirelles *et al.*, 2015; Birnbaum & Gerardo, 2016). Of these, undoubtedly the most celebrated are the 52 described species of leaf-cutting ants in the genera *Acromyrmex* Mayr and *Atta* Fabricius (Hölldobler & Wilson, 1990; Hölldobler & Wilson, 2011; Rabeling *et al.*, 2015). Whereas all other fungus-growing ants cultivate their fungus gardens on insect frass, flower parts, seeds, dry grass or other organic matter, the leaf-cutter species are unique in cutting fresh vegetation as the primary substrate for their fungus gardens. Their propensity to harvest large quantities of fresh leaves or grasses has made leaf-cutter ants the dominant herbivores in many Neotropical ecosystems as well as major pests of human agriculture (Cherrett, 1989; Hölldobler & Wilson, 1990; Della Lucia, 2011; Hölldobler & Wilson, 2011). In addition to growing their fungus gardens on freshly cut vegetation, leaf-cutter ants differ from other fungus-growing ants in having dramatically larger colony sizes (upwards of five million workers in *Atta*), increased female-caste polymorphism, increased queen mating frequencies, and, in *Atta*, claustral nest founding (Weber, 1972; Hölldobler & Wilson, 1990; Schultz & Brady, 2008; Mehdiabadi & Schultz, 2010; Fernández *et al.*, 2015; Mueller *et al.*, 2018).

Although leaf-cutting ants have long been the focus of natural history studies and coevolutionary research, their evolutionary origins remain somewhat enigmatic. In part, this is because their closest living relatives are a diverse and elusive group of ants in the genus *Trachymyrmex* (Weber, 1972; Brandão & Mayhé-Nunes, 2007; Rabeling *et al.*, 2007a; Schultz & Brady, 2008). As currently described, *Trachymyrmex* consists of 49 extant species, many of which are poorly known, and additional, undescribed species are known to exist. Although the taxonomy of this group has been challenging, revisions initiated by Walter W. Kempf and continued by Antonio J. Mayhé-Nunes and C. Roberto F. Brandão (Mayhé-Nunes & Brandão, 2002, 2005; Brandão & Mayhé-Nunes, 2007) have so far treated three narrowly defined species groups [*opulentus* (Mann), *iheringi* (Emery), and *jamaicensis* (André); 16 species in total] based on external morphology. Rabeling *et al.* (2007a) revised the North American species (nine additional species). Kempf referred to loosely defined *urichii* (Forel), *septentrionalis* (McCook), *cornetzi* (Forel) and *farinosus* (Emery) groups (see Brandão & Mayhé-Nunes, 2007). One prior phylogeny, resulting from a maximum-parsimony analysis of 50 morphological characters of the worker caste (Brandão & Mayhé-Nunes, 2007), supports the monophyly of *Trachymyrmex*. By contrast, a phylogeny based on larval morphology (Schultz & Meier, 1995), three multilocus molecular phylogenies (Schultz & Brady, 2008; Sosa-Calvo *et al.*, 2013, 2018), and four genome-scale phylogenies (Nygaard *et al.*, 2016; Branstetter *et al.*, 1975; Ješovnik *et al.*, 2017; Li *et al.*, 2018) all reconstruct *Trachymyrmex* as a paraphyletic grade in which the ancestor of the sister genera *Xerolitor* and *Sericomyrmex*, as well as the ancestor of the leaf-cutting genera *Atta* and *Acromyrmex*, arise from within a paraphyletic *Trachymyrmex*.

Elucidating the evolutionary relationships among *Trachymyrmex* ants and their associated fungi is key to understanding two evolutionary transitions in the evolution of attine agriculture. First, because the most recent common ancestor of all higher-attine ant genera (i.e. *Sericomyrmex*, *Xerolitor*, *Trachymyrmex*, *Atta* and *Acromyrmex*) was almost certainly *Trachymyrmex*-like, a detailed understanding of the evolutionary history of *Trachymyrmex* could provide some insight into the origin of higher-attine agriculture *c.* 30 Ma (Mueller & Rabeling, 2008; Schultz & Brady, 2008; Solomon *et al.*, 2011; Branstetter *et al.*, 1975). Notably, this transition appears to be correlated with the domestication of a particular lineage of

fungal cultivar, as indicated by: (i) the obligate dependence of higher-attine fungi on their ant hosts, in contrast to the lower-attine fungi, which are facultative symbionts, i.e. which retain population-genetic links to free-living populations; (ii) the consistent presence of gongylidia, swollen fungal hyphal tips that are preferentially harvested by the ants for food (Möller, 1893; Wheeler, 1907; Schultz & Brady, 2008; Mehdiabadi & Schultz, 2010; Masiulionis *et al.*, 2014; Schultz *et al.*, 2015); (iii) polyploidy, in contrast to lower-attine fungi, which are, so far as we know, consistently diploid (Kooij *et al.*, 2015; Carlson *et al.*, 2017); and (iv) increased expression of plant-degrading and detoxifying enzymes (De Fine Licht & Boomsma, 2010, 2014; De Fine Licht *et al.*, 2013; Nygaard *et al.*, 2016).

Second, because the ancestor of the leaf-cutting ant genera (i.e. *Atta* and *Acromyrmex*) was also almost certainly *Trachymyrmex*-like (Mueller & Rabeling, 2008; Schultz & Brady, 2008; Branstetter *et al.*, 1975), understanding the evolutionary history of *Trachymyrmex* could also provide insights into the origin of the leaf-cutting ants. Indeed, because *Trachymyrmex* is not monophyletic, but is instead a paraphyletic grade, reconstructing the evolutionary history of *Trachymyrmex* is an exercise in reconstructing the stepwise evolution that resulted in the origin of the leaf-cutting ants. Several *Trachymyrmex* species have morphological and behavioural traits that appear to be intermediate between generalized higher-attine agriculture and leaf-cutter agriculture (e.g. weak worker–worker polymorphism, occasional cutting and use of fresh vegetation as a fungal substrate, and, in some species, cultivating the same fungi as leaf-cutting ants), suggesting that some of the characteristics of leaf-cutter agriculture may have preceded others (Weber, 1972; Mueller & Rabeling, 2008; Schultz & Brady, 2008; Mehdiabadi & Schultz, 2010). Reconstructing the sequence of events that gave rise to the leaf-cutter syndrome would help to shed light on one of the most significant evolutionary innovations in the history of the fungus-growing ants, and arguably among insects more broadly.

In addition to the leaf-cutters, two other genera of fungus-growing ants arise from within the *Trachymyrmex* grade. One of these, *Sericomyrmex*, was recently revised by Ješovnik & Schultz (2017) and a phylogenomic analysis provided new insights into its coevolutionary history with its fungal cultivars (Ješovnik *et al.*, 2017). The other non-leaf-cutting lineage derived from *Trachymyrmex* consists of a single described species, *Xerolitor explicatus* (Kempf), which is the poorly known sister group to *Sericomyrmex* (Sosa-Calvo *et al.*, 2018). Given that the leaf-cutting ants have been relatively well studied (although a much-needed systematic revision of *Acromyrmex* is currently in preparation by C. Rabeling *et al.*) and that the other diverse clade of higher attines, *Sericomyrmex*, has now been taxonomically revised, *Trachymyrmex* remains the sole group of higher-attine ants for which critical information for many species is lacking.

To date, biological studies of *Trachymyrmex* have been limited to a small subset of species and are highly clustered geographically, focusing on a few species from the U.S.A., southern Central America (Panama and Costa Rica), and southeastern Brazil (Wheeler, 1907, 1911; Weber, 1972; Gonçalves, 1975; Villesen *et al.*, 1999; Villesen *et al.*, 2002; Currie *et al.*, 2003a; Seal & Tschinkel, 2006; Brandão & Mayhé-Nunes, 2007; Rabeling *et al.*, 2007a; Seal & Tschinkel, 2007a; Seal & Tschinkel, 2007b; Seal & Tschinkel, 2007c; Seal & Tschinkel, 2008; De Fine Licht & Boomsma, 2014; Sánchez-Peña *et al.*, 2017; Albuquerque *et al.*, 2018; Gray *et al.*, 2018). Yet *Trachymyrmex* ants occupy the widest native geographical distribution of any genus of fungus-growing ants – from Long Island, New York, in the north (40.9°N) to the pampas of Argentina in the south (37.5°S). The greatest diversity of *Trachymyrmex* species is in tropical South America, the area that has been least studied (Weber, 1972; Gonçalves, 1975; Mayhé-Nunes & Jaffé, 1998; Brandão & Mayhé-Nunes, 2007; Rabeling *et al.*, 2007a).

Consequently, the goal of this project was to elucidate the described evolutionary transitions by reconstructing the phylogenetic relationships among the known species of *Trachymyrmex* ants and their fungal cultivars. A second goal was to update the taxonomy of this problematic group of ants so that it is better aligned with the group's evolutionary history. To accomplish these goals, we sought to expand upon the taxonomic sampling of previous studies through targeted field collections as well as samples in existing collections and to reconstruct phylogenies for both ants and fungi using molecular markers.

We describe here the most comprehensive analysis to date of the evolutionary history of *Trachymyrmex* ants and their fungal cultivars. Our analyses are based on extensive sampling throughout the geographic range of the genus. In particular, our sampling focuses on the regions that, until now, have received comparatively less attention from fungus-growing ant researchers, including the greater Amazon Basin, the Cerrado and Caatinga ecosystems of Brazil, and the Guiana Shield.

Materials and methods

Field work

Samples included in this study were collected between 1991 and 2009 and span 13 countries: Argentina, Costa Rica, Cuba, Brazil, Ecuador, French Guiana, Guyana, Mexico, Panama, Peru, Suriname, Trinidad, and the U.S.A. (Fig. 1). The majority of targeted collections for this project took place in Brazil between 2008 and 2009. We selected additional specimens for analysis from existing collections in order to fill taxonomic gaps within *Trachymyrmex* and as outgroups. Table S1 lists all the specimens included in phylogenetic analyses.

Collections included, whenever possible, nest series consisting of workers, brood, alate males and gynes, and the queen. Natural history observations, including habitat type, external nest appearance, foraging substrate and nest architecture [chamber depth, dimensions and other features, as described in Rabeling *et al.* (2007b) and Sosa-Calvo *et al.* (2015)] were noted when possible. Voucher specimens are deposited in the Museu de Zoologia da Universidade de São Paulo, São Paulo, Brazil (MZUSP); the U.S. National Museum of Natural History in Washington, DC (USNM); and the Social Insect Biodiversity Repository at ASU in Tempe, AZ (SIBR).

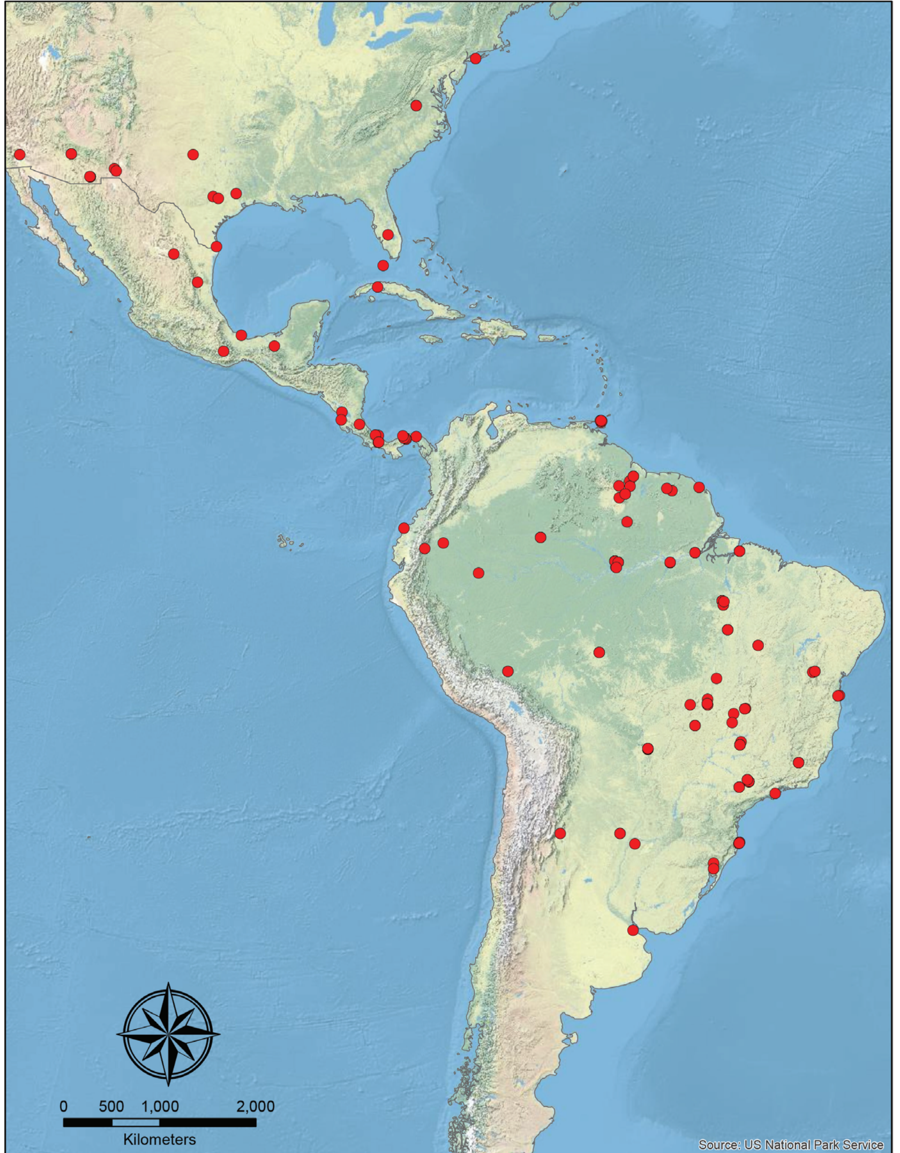

Fig. 1. Map of the Americas indicating locations sampled for ant and fungal specimens used in the phylogenetic analyses.

In our targeted collections of *Trachymyrmex*, we attempted to collect samples of fungus gardens from nests using a sterile technique (described in Sosa-Calvo *et al.*, 2015). Cultivar samples were collected in replicate such that some samples were placed directly into 95% ethanol while others were plated directly onto sterilized potato dextrose agar medium and further isolated and cultured at the Center for the Study of Social Insects laboratory at UNESP in Rio Claro, Brazil. Table S1 lists all specimens included in phylogenetic analyses of fungal cultivars.

DNA extraction and sequencing

Ant genomic DNA was extracted using the Qiagen DNeasy Tissue Kit (Qiagen, Valencia, CA, U.S.A.) following the manufacturer's protocols. Cell lysis with 20 μL of proteinase K was performed over a 24 h period (differing from manufacturer's protocol, which calls for 1–8 h) followed by several purification steps in spin mini-columns. The extracted DNA was eluted from the spin mini-column in two steps, each employing 50 μL of nuclease-free water (differing from the Qiagen procedure, which calls for 200 μL of AE buffer).

DNA was extracted primarily from adult worker ants. DNA was extracted destructively or nondestructively depending on the number of nest-series specimens available. DNA was destructively sampled from individuals that were subsets of nest series (i.e. entire or partially preserved colonies consisting of multiple developmental stages). In such cases, the whole ant specimen was dried for 30 min on a Kimwipe (Kimberly-Clark, Irving, TX, U.S.A.), placed in a 1.5 mL tube with a new or previously sterilized stainless-steel bead, and then finely pulverized at

Table 1. Primers used for sequencing mitochondrial (*cytochrome oxidase I*, *COI*) and nuclear (*elongation factor 1a F1 copy*, *EF1aF1*; *elongation factor 1a F2 copy*, *EF1aF2*; *long-wavelength rhodopsin*, *LW Rh*; and *wingless*, *wg*) gene fragments in ants and the nuclear ribosomal internal transcribed spacer (ITS) and elongation factor 1a (EF1a) regions in the fungal cultivars.

Gene region	Primer	Sequence 5′ to 3′	Source
Ants			
COI	LCO1490	GGT CAA CAA ATC ATA AAG ATA TTG G	Folmer *et al.* (1994)
	HCO2198	TAA ACT TCA GGG TGA CCA AAA AAT CA	Folmer *et al.* (1994)
	Jerry	CAA CAY TTA TTT TGA TTT TTT GG	Simon *et al.* (1994)
	Ben3R	GC WAC WAC RTA ATA KGT ATC ATG	Brady *et al.* (2000)
EF1a-F1	M3 F1-383F	CAT ATW AAC ATT GTS GTS ATY GG	Schultz & Brady (2008)
	10R F1-1887R	ACG GCS ACK GTT TGW CKC ATG TC	Schultz & Brady (2008)
	for2 F1-494F	AAG GAG GCT CAG GAG ATG GG	Schultz & Brady (2008)
	rev1 F1-1044R	CGT CTT ACC ATC GGC ATT GCC	Schultz & Brady (2008)
	U377 F1-792F	TT GGC GTG AAG CAG CTG ATC G	Schultz & Brady (2008)
	TRS1R F1-1189R	ACC TGG TTT YAA GAT RCC GGT	Schultz & Brady (2008)
	U52.1 F1-1109F	CCG CTT CAG GAT GTC TAT AA	Schultz & Brady (2008)
	L53 F1-1551R	CCG CGT CTC AGT TCY TTC AC	Mueller *et al.* (2006)
	TRS4F F1-1424F	GCG CCK GCG GCT CTC ACC ACC GAG G	Brady *et al.* (2006)
	TRS9R F1-1829R	GGA AGG CCT CGA CGC ACA TMG G	Brady *et al.* (2006)
EF1a-F2	515F	GGT TCC TTC AAR TAY GCY TGG GT	P.S. Ward, personal communication
	1371R	CC RAT CTT RTA YAC GTC CTGC	P.S. Ward, personal communication
	557F	GAA CGT GAA CGT GGT ATY ACS AT	Brady *et al.* (2006)
	1118R	TTAC CTG AAG GGG AAG ACG RAG	Brady *et al.* (2006)
LW Rh	LR134F	ACM GTR GTD GAC AAA GTK CCA CC	P.S. Ward, personal communication
	LR143F	GAC AAA GTK CCA CCR GAR ATG CT	Ward & Downie (2005)
	LR639ER	YTTAC CG RTT CCA TCC RAA CA	Ward & Downie (2005)
wg	Wg254F	CGA GAG ACC GCK TTY RTC TAY GC	P.S. Ward, personal communication
	Wg1038R	CA CTT NAC YTC RCA RCA CCA RTG	P.S. Ward, personal communication
	Wg290F	GCW GTR ACT CAC AGY ATC GC	P.S. Ward, personal communication
	wg645R	CG RTC CTT BAG RTT RTC GCC	P.S. Ward, personal communication
	Wg503F	CT CTC TCR TTA CAG CAC GT	Schultz & Brady (2008)
	Wg524EF	GCA GCA CGT TTC YTC VGA RAT GCG	P.S. Ward, personal communication
	Wg578F	TGC ACN GTG AAR ACY TGC TGG ATG CG	Ward & Downie (2005)
	wg1032R	AC YTC GCA GCA CCA RTG GAA	Abouheif & Wray (2002)
Fungi			
ITS	ITS1	TCC GTA GGT GAA CCT GCG G	White *et al.* (1990)
	ITS5	GGA AGT AAA AGT CGT AAC AAG G	White *et al.* (1990)
	5.8SF	TCG ATG AAG AAC GCA GC	Vilgalys & Hester (1990)
	5.8SR	CGC TGC GTT CTT CAT CG	Vilgalys & Hester (1990)
	ITS4	TCC TCC GCT TAT TGA TAT GC	White *et al.* (1990)
EF1a	EF1a-Fwd	GTT GCT GTC AAC AAG ATG GAC ACT AC	Mikheyev *et al.* (2006)
	EF1a-Rev	GCC TTG ATG ATA CCA GTC TCG ACA CG	Mikheyev *et al.* (2006)

25.0 Hz for 30 s using a Qiagen TissueLyser RETSCH MM200 in order to rapidly disrupt cells and tissues.

Fungal tissue used for DNA sequencing was manually separated from garden substrate using a dissecting microscope and extracted following a standard Chelex protocol (Sigma-Aldrich, St Louis, MO, U.S.A.).

DNA sequences were amplified by PCR in 25 μL solutions containing 1 μL of template DNA, 1 μL of each primer (forward and reverse), 12.2 μL of H_2O, 5 μL of 5x buffer, 2 μL of dNTPs, 2.5 μL of $MgCl_2$, and 0.3 μL of Taq polymerase (Promega, Maddison, WI, U.S.A.) or in 20 μL solutions containing 1 μL of template, 0.8 μL of each primer, 5.4 μL of H_2O, and 10 μL of PCR Master Mix (1.5 mm $MgCl_2$, 0.2 mm of dNTPs, and 1 unit of Taq polymerase) (Promega).

Polymerase chain reaction amplifications for all genes were performed in a thermal cycler programmed to run the following protocol: 1 min denaturation at 95°C; 34 cycles of 30 s denaturation at 95°C, 1 min annealing at 45–60°C (depending on primer set), and 1.5 min extension at 72°C; 1.5 min final extension at 72°C; and unlimited hold at 4–10°C.

Polymerase chain reaction products were visualized on ethidium-bromide-stained agarose gels (50 mL of 1.5% TBE gel – Tris/borate/EDTA – and 1 μL of ethidium bromide) by running 5 μL of product mixed with 1.5 μL of 6X loading dye for *c*. 30 min at 100 V. PCR product was purified by adding 3 μL of the enzymatic clean-up reagent ExoSAP-IT® (exonuclease I and shrimp alkaline phosphatase; Affymetrix Inc., Santa Clara, CA, U.S.A.), previously diluted in nuclease-free water (9:1), into the remaining 15–20 μL of PCR product. The solution was placed in a thermal cycler for 30 min at 37°C to allow the enzyme to remove unincorporated nucleotides and primers, followed by 15 min at 80°C, for enzyme deactivation. The

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primers employed in both amplification and sequencing are listed in Table 1. Sequencing reactions used 1 μL of the cleaned PCR product.

Sequencing of ant DNA was performed in the Laboratories of Analytical Biology of the National Museum of Natural History, Smithsonian Institution, on an ABI 3100 automated sequencer using an ABI BigDye Terminator v.3.1 Cycle Sequencing kit (Applied Biosystems Inc., Foster City, CA, U.S.A.). Fungal DNA sequences were generated in the Mueller Lab at The University of Texas at Austin on an ABI 3100 DNA Analyzer or an ABI 3730 DNA Analyzer (Applied Biosystems) at the ICMB Core Sequencing Facility of The University of Texas at Austin (icmb.utexas.edu/dna-sequencing-facility). Sequence data were assembled and edited using the program SEQUENCHER v.4.10.1 (Gene Codes Corp., Ann Arbor, MI, U.S.A.).

For this study, new genetic data were obtained from 112 ants (402 new gene sequences) and 95 fungi (153 new gene sequences). DNA sequences are deposited in GenBank under accession numbers MK599980–MK600381 for the ants and MK685681–MK685833 for the fungi (Tables S2, S3).

Data analyses

Ant DNA sequences consisted of fragments of one mitochondrial gene, *cytochrome c oxidase I* (*mtCOI*), and four nuclear genes, *EF1-alpha F1* (*EFF1*), *EF1-alpha F2* (*EFF2*), *wingless* (*WG*) and *long-wavelength rhodopsin* (*LWRh*). Gene fragments were individually aligned using MAFFT v.7.017 (Katoh *et al.*, 2002, 2005; Katoh & Standley, 2013) under the 'Auto' default algorithm as implemented in GENEIOUS R9 v8.1.8 (Kearse *et al.*, 2012), then error-checked and refined by eye in MESQUITE 3.11 (Maddison & Maddison, 2015). The aligned gene fragments were concatenated in MESQUITE 3.11 to produce two datasets: (i) a five-gene dataset, containing all five genes and 3775 total sites; and (ii) a four-gene data set, containing the four nuclear genes and 3117 total sites, but excluding *mtCOI*. For the five-gene data set, data were partitioned and modeled using the program PARTITIONFINDER v.1.1.1 (Lanfear *et al.*, 2012) under the Bayesian information criterion (BIC) with 18 data blocks consisting of: (i) the first-, second-, and third-codon positions of the coding regions of each of the five gene fragments (15 data blocks); and (ii) the introns from *EFF1 F1*, *LWRh*, and *WG* (three data blocks), and with a user tree resulting from an unpartitioned maximum likelihood best-tree analysis conducted in RAXML v.8.2.4 (Stamatakis, 2006). PARTITIONFINDER analyses of the four-gene dataset, which lacked the three data blocks from *mtCOI*, included 15 data blocks but were otherwise identically structured.

Fungal DNA sequences consisted of fragments of two nuclear genes, *internal transcribed spacer* (*ITS*) and *elongation factor 1-alpha* (*EF*). Fungal *ITS* gene sequences were aligned as described in Mehdiabadi *et al.* (2012) and Masiulionis *et al.* (2014). Fungal *EF* gene sequences were aligned under the 'Auto' default algorithm as implemented in GENEIOUS R9 v.8.1.8 (Kearse *et al.*, 2012), then further refined by eye in MESQUITE 3.11. The separate *ITS* and *EF* alignments were concatenated to produce an alignment consisting of 153 taxa and 1385 sites. For the concatenated dataset, data were partitioned and modelled using the program PARTITIONFINDER v.1.1.1 under the BIC with seven data blocks and with a user tree resulting from an unpartitioned maximum likelihood best-tree analysis conducted in RAXML v.8.2.4 (Stamatakis, 2006). Three data blocks consisted of the first-, second-, and third-codon positions of *EF*. As described in Mehdiabadi *et al.* (2012) and Masiulionis *et al.* (2014), *ITS* was divided into four data blocks of contiguous sites based on degree of variability: (i) ITSslow1 (sites 1–36); (ii) ITSfast1 (sites 37–471); (iii) ITSslow2 (sites 472–630); and ITSfast2 (sites 631–1062).

For both the ant and fungal datasets, the partitions and models identified by PARTITIONFINDER (Tables 2–4) were employed in both Bayesian and maximum likelihood analyses. Bayesian analyses used the MPI version of MRBAYES 3.2.5 (Ronquist *et al.*, 2012) on the Smithsonian Institution AntLab Atom-Ant cluster, with nucmodel = 4by4, nruns = 2, nchains = 8, samplefreq = 1000, and 20 million generations, with a burn-in of 2 million generations. To address known problems with branch-length estimation in MRBAYES (Marshall *et al.*, 2006; Rabeling *et al.*, 2008; Brown *et al.*, 2010; Marshall, 2010), we set brlenspr = unconstrained:Exp (100). Burn-in, convergence, and stationarity were assessed using TRACER v.1.5 (Rambaut *et al.*, 2018), by examining potential scale reduction factor values and .stat output files in MRBAYES, and by using Bayes factor comparisons of harmonic-mean marginal likelihoods of pairs of runs with standard error estimated using 1000 bootstrap pseudoreplicates in TRACER v.1.5, which employs the weighted likelihood bootstrap estimator of Newton & Raftery (1994) as modified by Suchard *et al.* (2001). Maximum likelihood analyses employed the MPI version of RAXML v.8.2.4 (Stamatakis, 2006) on the Smithsonian Institution AntLab's AntPAC cluster, with simultaneous rapid bootstrap (1000 replicates) and best-tree analyses.

Both the ant and fungal datasets are deposited in Dryad (DOI: doi:10.5061/dryad.2p7r771).

Results and Discussion

Ant and fungal phylogenetics

In terms of taxon representation, the phylogenies we reconstructed for *Trachymyrmex* ants (Fig. 2) and their fungal cultivars (Fig. 3) constitute the most comprehensive analyses to date of the evolutionary history of this group of fungus-growing ants. Our results are consistent with previous molecular phylogenies that included *Trachymyrmex* (Schultz & Brady, 2008; Sosa-Calvo *et al.*, 2013, 2018; Ješovnik *et al.*, 2016; Branstetter *et al.*, 1975; Li *et al.*, 2018) in the following ways. First, *Trachymyrmex*, as currently defined, is paraphyletic with respect to the clade that contains the leaf-cutter ants (*Acromyrmex* and *Atta*) and the social parasite genus *Pseudoatta* Gallardo (C. Rabeling, in preparation). Second, *Trachymyrmex*, as

Table 2. The five data subset partitions and models identified in a PARTITIONFINDER 1.1.1 analysis of the four-nuclear-gene dataset for the ants, consisting of the coding regions of *EF1-alpha F1* (*F1*), *EF1-alpha F2* (*F2*), *wingless* (*wg*) and *long-wavelength rhodopsin* (*LWRh*), and the introns of *F1*, *wg* and *LWRh*.

Subset	Best model	Subset partitions
1	TrN + I + G	F1_pos1, F2_pos1, wg_pos2
2	F81 + I	F1_pos2, F2_pos2
3	K80 + G	F1_pos3, F2_pos3, LWRh_pos3, wg_pos3
4	K80 + I + G	LWRh_pos1, LWRh_pos2, wg_pos1
5	TVMef	F1_intron, LWRh_intron, wg_intron

Input included 15 data blocks consisting of the first-, second-, and third-codon positions of the coding regions and the three introns, as well as a user tree resulting from an unpartitioned maximum likelihood analysis conducted in RAXML v.8.2.4.

Table 3. The seven data subset partitions and models identified in a PARTITIONFINDER 1.1.1 analysis of the five-gene dataset for the ants, consisting of the coding regions of mitochondrial *cytochrome c oxidase I* (*COI*); the nuclear genes *EF1-alpha F1* (*F1*), *EF1-alpha F2* (*F2*), *wingless* (*wg*) and *long-wavelength rhodopsin* (*LWRh*); and the introns of *F1*, *wg* and *LWRh*.

Subset	Best model	Subset partitions
1	GTR + I + G	F1_pos1, F2_pos1, wg_pos1, wg_pos2
2	GTR + I + G	F1_pos2, F2_pos2
3	GTR + G	F1_pos3, F2_pos3, LWRh_pos3, wg_pos3
4	GTR + I + G	COI_pos2, LWRh_pos1, LWRh_pos2
5	GTR + I + G	COI_pos3
6	GTR + G	COI_pos1
7	GTR + G	F1_intron, LWRh_intron, wg_intron

Input included 18 data blocks consisting of the first-, second-, and third-codon positions of the coding regions and the three introns, as well as a user tree resulting from an unpartitioned maximum likelihood analysis conducted in RAXML v.8.2.4.

Table 4. The four data subset partitions and models identified by PARTITIONFINDER 1.1.1 analysis of the two-gene dataset for the fungi, consisting of *EF1-alpha* (*EF*) and *internal transcribed spacer* (*ITS*).

Subset	Best model	Subset partitions
1	K80 + I + G	EFpos1, EFpos2, ITSslow1
2	TrNef + G	EFpos3
3	TVM + I + G	ITSfast1, ITSfast2
4	K80 + I	ITSslow2

Input included seven data blocks consisting of the first-, second-, and third-codon positions of *EF* and four contiguous blocks of sites in *ITS* differentiated by the variability, as well as a user tree resulting from an unpartitioned maximum likelihood analysis conducted in RAXML v.8.2.4.

currently defined, is paraphyletic with respect to the clade that contains *Sericomyrmex* and *Xerolitor explicatus* (the latter of which was not included in this analysis but has been shown in previous studies to be the sister group of *Sericomyrmex*; Branstetter *et al.*, 1975; Ješovnik *et al.*, 2017; Li *et al.*, 2018; Sosa-Calvo *et al.*, 2018). Third, as currently defined, *Trachymyrmex* is a grade that consists of three clades, each of which is well supported by Bayesian posterior probabilities and maximum likelihood bootstrap proportions (Fig. 2).

Based on the improved taxon sampling in our study, the robust support for all three clades, and the fact that previous studies with more incomplete taxon sampling reconstructed the same three clades (Schultz & Brady, 2008; Sosa-Calvo *et al.*, 2013, 2018; Ješovnik *et al.*, 2016; Nygaard *et al.*, 2016; Branstetter *et al.*, 1975; Ješovnik *et al.*, 2017; Li *et al.*, 2018), we conclude that these three clades correspond to three distinct genera of ants. The sister clade to all other higher-attine ants contains *Sericomyrmex* and *Xerolitor* (Fig. 2; position of *Xerolitor* not shown) as well as 30 described species (Table 5) formerly assigned to *Trachymyrmex*, described herein as *Mycetomoellerius* **gen.n.** (Fig. 2). Half of the species of *Mycetomoellerius* occur in seasonally dry habitats of South America and the remainder occur in Central America, the Caribbean, and the southern U.S.A. The most recently derived clade is the genus *Trachymyrmex* s.s., which is the sister clade of the leaf-cutting ants (*Atta*, *Acromyrmex* and *Pseudoatta*) and contains nine described species (Table 5), eight of which are restricted to North America and/or northern Mexico and one of which, *T. saussurei* (Forel), is known to occur from southern Mexico to Honduras. The third clade, *Paratrachymyrmex* **gen.n.** (Fig. 2), the sister clade of *Trachymyrmex* s.s. and the leaf-cutting ants, contains nine described species that primarily occur in wet forest habitats in northern South America and Central America.

Based on morphological differences as well as on robust branch support for distinct lineages, our analyses suggest the existence of at least 11 new species (Fig. 2). Of these, eight belong to *Mycetomoellerius* and two belong to *Paratrachymyrmex*. Species-level taxonomic work on *Mycetomoellerius* and *Paratrachymyrmex* is required to fully resolve the status of these species and to formally describe them.

Our phylogenetic analyses of the fungi cultivated by *Trachymyrmex*, *Mycetomoellerius* and *Paratrachymyrmex* offer several new insights into the coevolutionary dynamics between higher-attine ants and their fungal cultivars. Numerous previous studies have shown that higher-attine fungi differ from lower-attine fungi in a number of ways. First, unlike all lower-attine fungi, which, so far as is known, are facultative symbionts capable of living freely apart from the ants (Mueller *et al.*, 1998; Vo *et al.*, 2009), higher-attine fungi are obligate symbionts that, to date, have never been found apart from their ant hosts (Schultz *et al.*, 2005; Mehdiabadi & Schultz, 2010; Mueller *et al.*, 2017, 2018). Second, higher-attine fungi consistently produce gongylidia, swollen hyphal tips that are preferentially harvested by the ants for food, whereas lower-attine fungi lack gongylidia, although, in rare cases, certain lower-attine fungi have been observed to express gongylidia-like structures (Möller, 1893; Urich, 1895; Weber, 1972; Mueller *et al.*, 2001; Schultz *et al.*, 2005; Masiulionis *et al.*, 2014). Third, unlike lower-attine fungi, which are, as far as we know, all diploid, all higher-attine fungi are polyploid (Scott *et al.*, 2009; Kooij *et al.*, 2015; Carlson *et al.*, 2017). Fourth, higher-attine fungi differ significantly from lower-attine fungi in chitin synthesis and levels of expression of plant-degrading and detoxifying enzymes

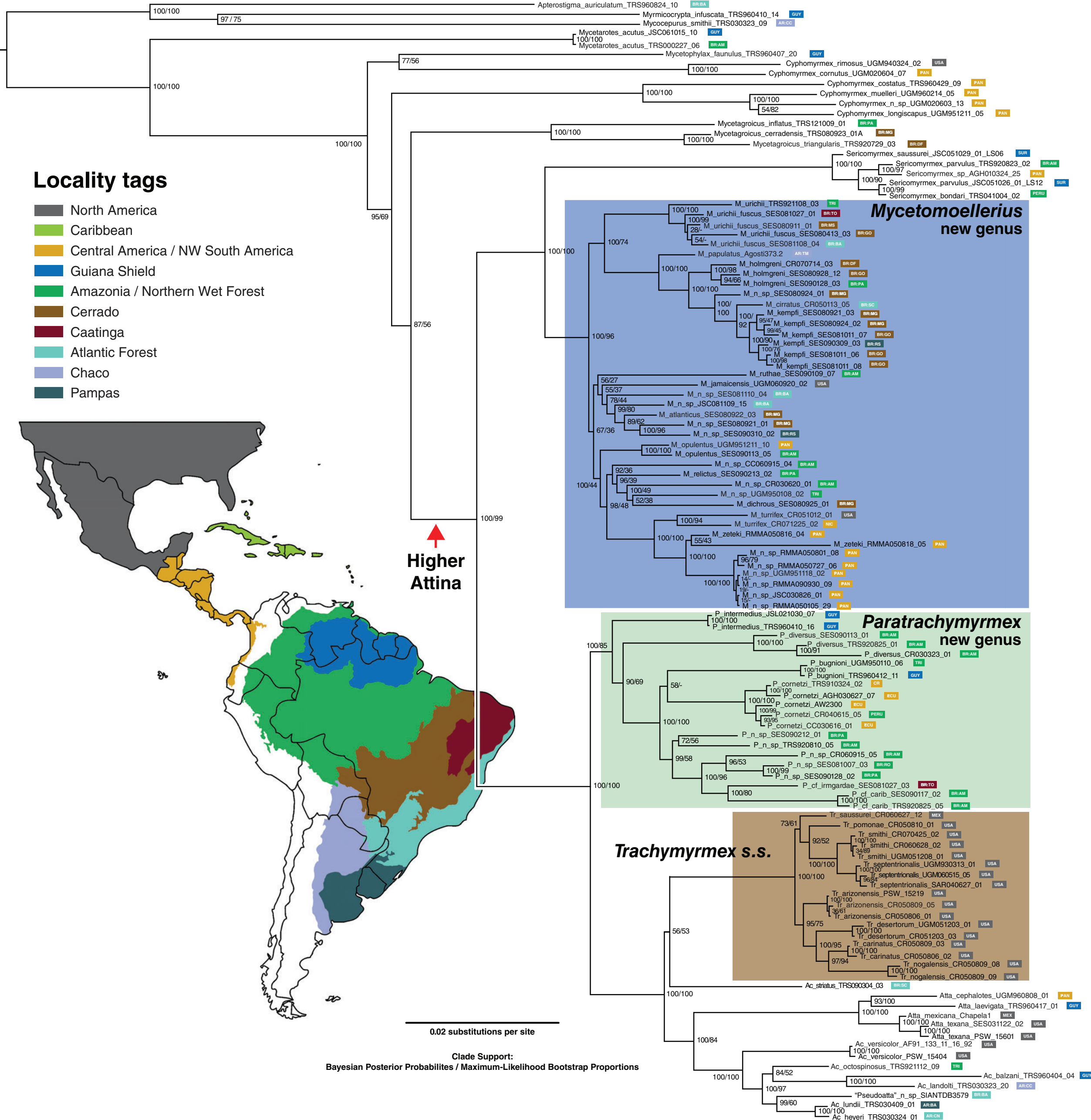

Fig. 2. Phylogeny of higher-attine ants resulting from Bayesian analysis of four nuclear gene fragments (see text for details). Bayesian posterior probabilities (PP) and maximum likelihood bootstrap (MLBS) proportions (resulting from maximum likelihood analysis; see text) are formatted as PP/MLBS. Clades formerly assigned to *Trachymyrmex* s.l. are indicated by the three large boxes. Tr, *Trachymyrmex* s.s.; Ac, *Acromyrmex*; M, *Mycetomoellerius* **gen.n.**; P, *Paratrachymyrmex* **gen.n.** Locality tags (small coloured boxes) to the right of taxon names indicate the ecological region of origin (by colour; see key), country of origin, and, for Brazil and Argentina, state or province of origin as follows: AR, Argentina (BA, Buenos Aires, CC, Chaco, CN, Corrientes, TM, Tucuman); BR, Brazil (AM, Amazonas, BA, Bahia, DF, Distrito Federal, GO, Goiás, MG, Minas Gerais, MS, Mato Grosso do Sul, MT, Mato Grosso, PA, Pará, RO, Rondônia, RS, Rio Grande do Sul, SC, Santa Catarina, SP, São Paulo, TO, Tocantins); CR, Costa Rica; ECU, Ecuador, GUY, Guyana; MEX, Mexico; NIC, Nicaragua; PAN, Panama; SUR, Suriname; TRI, Trinidad; USA, United States of America. The position of *Acromyrmex striatus* as the sister to *Trachymyrmex* s.s. is weakly supported and should be ignored; it has been shown instead to be the sister of the rest of the leaf-cutting ants, i.e. of *Acromyrmex* s.s. + *Atta* (Cristiano *et al.*, 2013; Branstetter *et al.*, 1975).

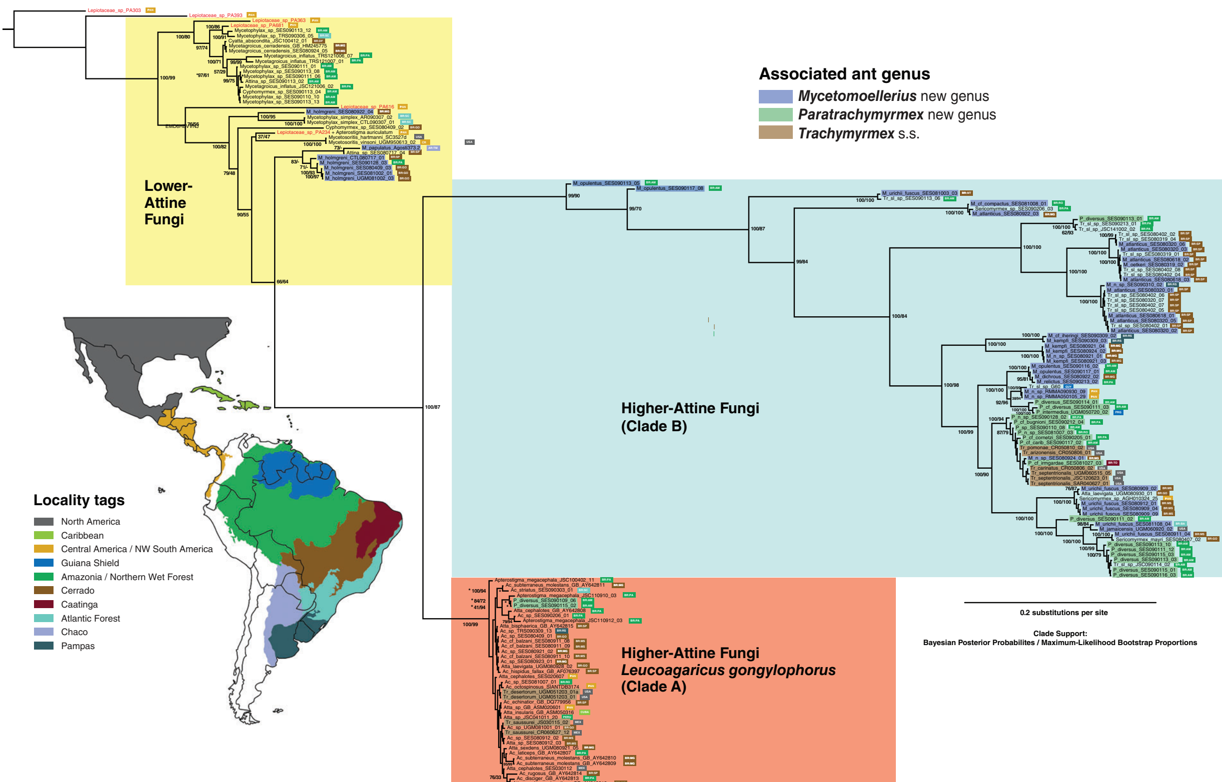

Fig. 3. Phylogeny of attine ant-associated fungi resulting from Bayesian analysis of two nuclear gene fragments (see text for details). Bayesian posterior probabilities (PP) and maximum likelihood bootstrap proportions (resulting from maximum likelihood analysis; see text) are formatted as PP/MLBS. Cases in which both values were < 70 are not reported. Lower-attine fungi are highlighted in yellow (with the names of free-living fungi in red); higher-attine fungi are highlighted in blue (clade B) and red (*Leucoagaricus gongylophorus*). Tr, *Trachymyrmex* s.s.; Ac, *Acromyrmex*; M, *Mycetomoellerius* **gen.n.**; P, *Paratrachymyrmex* **gen.n.**; and Tr_sl, *Trachymyrmex* s.l. Colours highlighting taxon names indicate associated *Trachymyrmex* s.l. ant genus; nonhighlighted '*Trachymyrmex* s.l. sp.' taxa were not identified to genus. Locality tags (small coloured boxes) to the right of taxon names indicate the ecological region and country of origin (see Fig. 2 caption for details).

(De Fine Licht & Boomsma, 2010, 2014; De Fine Licht *et al.*, 2013; Nygaard *et al.*, 2016).

The higher-attine fungi can be divided into two sister clades (Fig. 3), one of which is arguably a single species, *Leucoagaricus gongylophorus* ('clade A' of Mueller *et al.*, 2017, 2018), largely (but not entirely) cultivated by leaf-cutting ants. The second clade ('clade B' of Mueller *et al.*, 2018) consists of multiple species, largely (but, again, not entirely) cultivated by non-leaf-cutting higher-attine ants. Our data provide new evidence of the cultivation of *L. gongylophorus* by non-leaf-cutting ants, indicating that at least three non-leaf-cutting species [*Paratrachymyrmex diversus* (Mann), *Trachymyrmex desertorum* (Wheeler), and *T. saussurei*] cultivate *L. gongylophorus* (Fig. 3). Data from another study (Mueller *et al.*, 2018) corroborate the cultivation of *L. gongylophorus* by *T. desertorum* and *T. saussurei*, and indicate that *L. gongylophorus* is also cultivated by *Mycetomoellerius opulentus*, *Paratrachymyrmex intermedius* (Forel) and *Trachymyrmex arizonensis* (Wheeler). Taken together, these species represent all three of the clades formerly included within *Trachymyrmex* s.l., suggesting that associations between *L. gongylophorus* and these ant species are not strictly correlated with ant phylogeny.

Furthermore, the associations between *L. gongylophorus* and these ant species do not appear to be correlated with habitats occupied by extant species. Three species (*T. desertorum*, *T. smithi* Buren and *T. nogalensis* Byers) occur in arid regions in North America (Rabeling *et al.*, 2007a), one (*T. saussurei*) occurs in wet tropical forests in Mexico and northern Central America (Rabeling *et al.*, 2007a), one (*M. opulentus*) occurs in wet forests from Guatemala to Amazonia (Mayhé-Nunes & Brandão, 2002), and one (*P. diversus*) is found in wet forests in northern South America (Mann, 1916; Kempf, 1972; Brandão, 1991). The only geographic correlate of these observed ant–fungus associations is that none of them occurs south of Amazonia. Four of these ant species, *P. diversus*, *M. opulentus*, *P. intermedius* and *T. arizonensis*, also cultivate clade B fungi (Fig. 3; Mueller *et al.*, 2018), suggesting that it may be possible that one or more of these species cultivates both *L. gongylophorus* and clade B fungi or, alternatively, that each of these four species may consist of two or more cryptic species, each of which consistently cultivates only one cultivar type (as discovered in the *Cyphomyrmex wheeleri* Forel group; Mehdiabadi *et al.*, 2012). Because our sample sizes are small, the frequencies with which a given ant species cultivates *L. gongylophorus*

Table 5. Species composition of genera formerly assigned to *Trachymyrmex* s.s. The asterisk (*) indicates the oldest name (i.e. the type species) in each genus.

Species	Author and year described
Mycetomoellerius **gen.n.**	
agudensis	Kempf, 1967
atlanticus	Mayhé-Nunes & Brandão, 2007
cirratus	Mayhé-Nunes & Brandão, 2005
compactus	Mayhé-Nunes & Brandão, 2002
dichrous	Kempf, 1967
echinus	Weber, 1938
farinosus	Emery, 1894
fiebrigi	Santschi, 1916
gaigei	Forel, 1914
guianensis	Weber, 1937
haytianus	Wheeler & Mann, 1914
holmgreni	Wheeler, 1925
*iheringi**	Emery, 1888
isthmicus	Santschi, 1931
ixyodus	Mayhé-Nunes & Brandão, 2007
jamaicensis	André, 1893
jamaicensis antiguensis	Weber, 1938; possibly a distinct species; see Mayhé-Nunes & Brandão, 2007
kempfi	Fowler, 1982
oetkeri	Forel, 1908
opulentus	Mann, 1922
papulatus	Santschi, 1922
primaevus (fossil)	Baroni-Urbani, 1980
pruinosus	Emery, 1906
relictus	Borgmeier, 1934
ruthae	Weber, 1937
squamulifer	Emery, 1896
tucumanus	Forel, 1914
turrifex	Wheeler, 1903
urichii	Forel, 1893
verrucosus	Borgmeier, 1948
zeteki	Weber, 1940
Paratrachymyrmex **gen.n.**	
bugnioni	Forel, 1912
carib	Weber, 1945
cornetzi	Forel, 1912
diversus	Mann, 1916
*intermedius**	Forel, 1909
irmgardae	Forel, 1912
levis	Weber, 1938
mandibularis	Weber, 1938
phaleratus	Wheeler, 1925
Trachymyrmex Forel 1893	
arizonensis	Wheeler, 1907
carinatus	Mackay & Mackay, 1997
desertorum	Wheeler, 1911
nogalensis	Byars, 1951
pakawa	Sanchez-Peña *et al.*, 2017
pomonae	Rabeling & Cover, 2007
saussurei	Forel, 1885
*septentrionalis**	McCook, 1881
smithi	Buren, 1944

versus clade B fungi remains unclear, suggesting that future research should concentrate on these interesting cases (*P. diversus*, *P. intermedius* and *T. arizonensis)* to determine whether cultivation of both *L. gongylophorus* and clade B fungi occurs within the same ant population.

Our results provide new evidence for the cultivation of lower-attine fungi by higher-attine ants. Previous observations of higher-attine ants cultivating lower-attine fungi included a nest of *Mycetomoellerius papulatus* (Santschi) (Mueller *et al.*, 1998, 2017, 2018) and two nests of *Mycetomoellerius iheringi* (Mueller *et al.*, 2018). Remarkably, we found that *Mycetomoellerius holmgreni* (Wheeler) consistently cultivates lower-attine fungi (Fig. 3). Five *M. holmgreni* nests, collected in three widely separated locations (in the Brazilian states of São Paulo, Goiás and Pará), were each found to cultivate what is probably the same species of lower-attine fungus, which is very closely related to the fungus cultivated by *M. papulatus* mentioned earlier. A sixth nest collected in Mato Grosso cultivated a different lower-attine fungus. Intriguingly, *M. holmgreni* was reported by Gonçalves (1975) to cut leaves of the grass *Paspalum ancylocarpum* Nees ex Steud. Mayhé-Nunes & Brandão (2005) likewise reported that grass clippings are used as a substrate for cultivating this species' fungus gardens and that grass clippings are also used to construct the turret that forms its nest entrance, an observation corroborated by Albuquerque *et al.* (2018). A detailed study of one population of *M. holmgreni* found that this species utilizes a range of foraging substrates, including herbivorous insect frass, grass seeds and small flowers (Lizidatti, 2006). Our analyses suggest that at least some higher-attine ant species, such as *M. holmgreni*, may consistently cultivate lower-attine fungi. It should be noted that we know of only one exception to the otherwise consistent opposite pattern, in which a lower-attine ant cultivates a higher-attine fungus, that of *Apterostigma megacephala* Lattke (Schultz *et al.*, 2015).

If the higher-attine fungi originated simultaneously with the higher-attine ants, and if the descendants of the ancestral higher-attine ant continued to cultivate descendants of the ancestral higher-attine fungus, then the cultivation of lower-attine fungi by *M. papulatus*, *M. iheringi* and *M. holmgreni* could be due to evolutionary reversals, i.e. to secondary reacquisitions of lower-attine fungi. Interpreting the history of ant–fungus associations based on extant patterns becomes more complicated, however, if the origins of higher-attine ants and of higher-attine fungi occurred at different times (Mueller *et al.*, 2017, 2018). Given that only a single case is known of a lower-attine ant cultivating a higher-attine fungus, and given that this association is almost certainly due to a secondary acquisition (Schultz *et al.*, 2015), it seems unlikely that higher-attine fungi arose prior to the origin of higher-attine ants. In fact, available estimates (summarized in Table 1 in Mueller *et al.*, 2017) for the origins of higher-attine fungi and higher-attine ants indicate that higher-attine fungi may have arisen about 4–10 Ma after the origin of higher-attine ants. This suggests that the earliest higher-attine ants must have cultivated other fungi for the first few Ma of higher-attine evolution and that the cultivation of lower-attine fungi by *M. papulatus*, *M. iheringi* and *M. holmgreni* could be due to the retention of the original fungicultural state of higher-attine ants.

Moreover, it has been argued that current ant–fungus associations may be due to mass replacements of some or most of the original ancestral fungal cultivars by much more recently evolved fungal cultivars (e.g. in the leaf-cutter ants; Mikheyev *et al.*, 2010). If such a replacement occurred in the higher-attine ants, then the cultivation of lower-attine fungi by *M. papulatus*, *M. iheringi* and *M. holmgreni* would likewise be interpreted as a retention of an ancestral association of higher-attine ants with lower-attine fungi or the retention of an ancestral plasticity for cultivating either lower- or higher-attine fungi (resulting in, for example, the cultivation of multiple fungal cultivars in the same ant population). Under this scenario, however, the original lower fungal cultivars of multiple other higher-attine ant species would have been replaced by higher cultivars, begging the question as to why this did not also occur in *M. papulatus*, *M. iheringi* and *M. holmgreni*. Although the evolutionary history and biological mechanisms remain unclear, the fact remains that the observed patterns of extant ant–fungus associations revealed by our ant and fungal phylogenies (Figs 2, 3) are, at the level of higher- versus lower-attine agriculture, largely but not entirely congruent with ant and fungal phylogenies. Among other explanations, such a pattern is consistent with a scenario in which some transitional states in fungiculture are retained because they are adaptive under specific conditions, causing their persistence for extended periods of time.

Mueller *et al.* (2017) demonstrated a similar pattern with regard to ant associations with *Leucoagaricus gongylophorus* and other higher-attine (Clade B) fungi, i.e. that they are largely but not entirely correlated with ant and fungal phylogenies. Although most non-leaf-cutting higher-attine ants (in the genera *Mycetomoellerius*, *Paratrachymyrmex*, *Xerolitor* and *Sericomyrmex*) cultivate non-*L. gongylophorus* (Clade B) higher-attine fungal species, and although most leaf-cutting ants (*Atta* and *Acromyrmex*) cultivate *L. gongylophorus*, exceptions continue to be discovered as sample sizes increase. Within each of these broad associations between groups of ants and fungi, there are no obvious phylogenetic correlations, i.e. at least in fungal clade B, which consists of multiple species, distantly related ants can cultivate the same fungal species and closely related ants can cultivate distantly related fungal species (Fig. 3; also Mueller *et al.*, 2018). This has led to the prevailing hypothesis of diffuse coevolution (Mikheyev *et al.*, 2007, 2010) for explaining the significant genetic evolution that has occurred in the higher-attine ants and fungi (Nygaard *et al.*, 2016). Nevertheless, at broad historical scales, i.e. at the level of the time periods separating lower agriculture from higher agriculture, and, within higher agriculture, separating *L. gongylophorus* from clade B fungi, there is a significant correlation between ant and fungal phylogenies consistent with associations persisting for millions of years. Additional work is needed to elucidate the evolutionary processes and biological mechanisms that gave rise to and maintain this pattern.

Experiments in which *Trachymyrmex* ants were raised on fungal cultivars isolated from leaf-cutter ant [*Atta texana* (Buckley)] nests show that at least some *Trachymyrmex* species (e.g. *T. septentrionalis*, *T. turrifex* (Wheeler) and *T. arizonensis*) are capable of cultivating *Leucoagaricus gongylophorus* to varying extents (Seal & Tschinkel 2007a; Seal & Mueller, 2014; Seal *et al.*, 2014). Some populations of *T. arizonensis* cultivate both *L. gongylophorus* and other higher-attine (clade B) fungi. Unless they represent cryptic species, both *Acromyrmex striatus* (Roger) and *Atta laevigata* (Smith) likewise cultivate *L. gongylophorus* and clade B fungi (Fig. 3; Mueller *et al.*, 2018). These cases provide support for the idea that higher-attine ants and their cultivars have not tightly coevolved.

Much work remains to be done in order to better characterize the biology of species of *Mycetomoellerius* and *Paratrachymyrmex*. The specialized fungal parasites in the genus *Escovopsis* Muchovej and Della Lucia (Ascomycota: Hypocreales), which are found only in association with attine nests and which compete with the ants to feed on the fungal cultivars, are only partially known for species in these genera. To date, studies have focused on *Escovopsis* species associated with four *Mycetomoellerius* species [*M. zeteki* (Weber), *M. ruthae* (Weber), *M. dichrous* (Kempf), *M. atlanticus* (Mayhé-Nunes and Brandão)] and one *Paratrachymyrmex* species (*P. diversus*) (Currie *et al.*, 1999a; Currie *et al.*, 2003a; Meirelles *et al.*, 2014, 2015; Birnbaum & Gerardo, 2016).

Likewise, few studies to date have included actinobacteria, which grow in cuticular crypts on the ants' integuments (Currie *et al.*, 1999b; Currie, 2001; Currie *et al.*, 2003c; Currie *et al.*, 2006; Cafaro *et al.*, 2011), isolated from *Mycetomoellerius* species. However, Meirelles *et al.* (2014) found several novel strains of *Pseudonocardia* isolated from previously unidentified *Trachymyrmex* s.l. species, several of which can now be identified as *M. atlanticus*. Several others remain unidentified but probably also belong to *Mycetomoellerius*. Given the diversity and phylogenetic position of *Mycetomoellerius*, future work on the microbiome of this genus is likely to reveal additional insights into the ecology and evolutionary history of these bacteria and their role in the higher-attine symbiosis (Currie *et al.*, 2003b; Currie *et al.*, 2003c; Currie *et al.*, 2006; Cafaro *et al.*, 2011; Mueller, 2012; Scheuring & Yu, 2012; Heine *et al.*, 2018).

By clarifying the phylogenetic relationships among higher-attine ants and their fungal cultivars and aligning genus-level ant taxonomy with evolutionary history, we hope to pave the way for additional studies on the evolutionary history of this system. Of particular interest are the origin of higher-attine agriculture and the origin of leaf-cutter agriculture (Mueller & Rabeling, 2008; Schultz & Brady, 2008; Mehdiabadi & Schultz, 2010; Nygaard *et al.*, 2016; Branstetter *et al.*, 1975). Within this context, outstanding questions that can now be approached more directly include the following:

1. *What was the timing of the origin of higher-attine agriculture?* Our discovery that some higher-attine ants cultivate lower-attine fungi is consistent with a scenario in which higher-attine fungi arose subsequent to the origin of higher-attine ants, displacing the ancestral cultivars in most but not all higher-attine ant species, as postulated for the leaf-cutting ants by Mikheyev *et al.* (2010). However, other interpretations for the timing of the origin of higher-attine agriculture remain possible, including a simultaneous origin of higher-attine ants and fungi. Since

the ancestor of the higher-attine fungi acquired many significant biological traits relative to those in lower-attine fungi (including obligate symbiosis, polyploidy, consistent expression of gongylidia, and significant changes in the expression of a number of key enzymes), each of these traits should be carefully examined in the fungi of *Mycetomoellerius*, *Sericomyrmex* and *Xerolitor* (Möller, 1893; Weber, 1972; Mueller, 2002; Scott *et al.*, 2009; De Fine Licht & Boomsma, 2010, 2014; Mehdiabadi & Schultz, 2010; De Fine Licht *et al.*, 2013; Kooij *et al.*, 2015; Nygaard *et al.*, 2016; Carlson *et al.*, 2017; Mueller *et al.*, 2018). A promising avenue of research would be to determine what traits (i.e. ant behaviours, ant microbiome composition, etc.) contribute to the success and persistence of ant–fungus associations in higher-attine agriculture, which could perhaps be pursued using controlled switches of particular ants, cultivars and associated microbes.

2. *What was the timing of the origin of leaf-cutter agriculture?* Now that the phylogenetic position of *Trachymyrmex* s.s. as the sister group of the leaf-cutting genera *Acromyrmex* and *Atta* is confirmed, and *Paratrachymyrmex* is established as the sister group of the clade containing both *Trachymyrmex* s.s. and the leaf-cutters, insight into the biology of *Paratrachymyrmex*, *Trachymyrmex*, *Acromyrmex* and *Atta* can help to elucidate the events associated with the major transition to leaf-cutting behaviour. These events include dramatic increases in colony size, increased female caste polymorphism, increased queen-mating frequency (i.e. polyandry) and a reliance on fresh vegetation as a substrate for fungal cultivation (Weber, 1972; Oster & Wilson, 1979; Hölldobler & Wilson, 1990, 2011; Villesen *et al.*, 1999; Murakami *et al.*, 2000; Villesen *et al.*, 2002; Schultz & Brady, 2008; Mehdiabadi & Schultz, 2010; Mueller *et al.*, 2018). Careful studies of the nesting biology, queen mating frequency, fungiculture and other aspects of the biology of *Mycetomoellerius*, *Paratrachymyrmex* and *Trachymyrmex* are lacking for many species and would provide insight into the order of events involved in this transition.
3. *What was the biogeographic context of the origin of leaf-cutter agriculture?* It is noteworthy that all *Trachymyrmex* s.s. species are North American in distribution, occurring in the U.S., Mexico, Honduras or Belize (Kempf, 1972; Rabeling *et al.*, 2007a). This suggests that the biogeographic context for the split between *Trachymyrmex* s.s. and the common ancestor of the leaf-cutter ants may have involved the separation of North America and South America prior to the formation of the Isthmus of Panama (O'Dea *et al.*, 2016). Consistent with this scenario is the fact that basally diverging lineages in both *Atta (Archaeatta* Gonçalves) and *Acromyrmex* [*A. versicolor* (Pergande)] also have northern distributions (Mexico/Cuba/southern U.S.). Inconsistent with this scenario, however, the species currently reconstructed as the sister group of all other leaf-cutting ants (contrary to our Fig. 2; see Branstetter *et al.*, 1975), *A. striatus*, has a southern South American distribution. Branstetter *et al.* (1975) found support for a dry-habitat, South American origin of all higher-attine ants (consistent with speculation by Kusnezov, 1963; Fowler, 1983) and for a dry-habitat, Mesoamerican origin of leaf-cutter ants. It is currently difficult to reconcile these hypotheses, especially given the distribution of *A. striatus*. Fossil-calibrated phylogenetic analyses of higher-attine ants that exhaustively sample all the species of *Paratrachymyrmex*, *Trachymyrmex*, *Acromyrmex* and *Atta* (currently in progress by C. Rabeling *et al.*) are the best strategy for reconstructing the biogeographic history of higher-attine agriculture.

Taxonomy

Given the large number of species sampled and the robust statistical support for the three clades described earlier (Fig. 2), and given that *Trachymyrmex* as currently defined is paraphyletic both with respect to *Xerolitor* and *Sericomyrmex* and with respect to *Atta* and *Acromyrmex*, we propose the following taxonomic actions.

Trachymyrmex *s.s. Forel, 1893*

Type species: *Trachymyrmex septentrionalis* (McCook, 1881)
Diagnosis:

1. Preocular carina curving mesad above the eye (Rabeling *et al.*, 2007a: figs 1B, 3B, 5B, 10B, 13B, 15B, 17B; Sánchez-Peña *et al.*, 2017: fig. 2).
2. Posterior margin of postpetiole with distinct concave emargination (Rabeling *et al.*, 2007: figs 1C, 3C, 5C, 10C, 12C, 15C, 17C; Sánchez-Peña *et al.*, 2017: fig. 2).
3. Eyes relatively small and strongly convex.
4. Mandibles distinctly striate; striae extending from lateral to masticatory margin.
5. Biogeographic distribution limited to North America, including Mexico and the United States, except for *T. saussurei* (Forel), which occurs in Mexico and northwestern Central America, and *T. turrifex* (Wheeler), which occurs in Nicaragua (Table S1).

Species composition: see Table 5.

Mycetomoellerius *Solomon, Rabeling, Sosa-Calvo and Schultz,* ***gen.n.***

Type species: *Mycetomoellerius iheringi* (Emery, 1894)
Diagnosis:

1. Preocular and frontal carinae subparallel, extending to the posterior cephalic corner to form a more or less distinct antennal scrobe (Mayhé-Nunes & Brandão, 2002: figs 2, 5, 8, 11, 14; Mayhé-Nunes & Brandão, 2005: figs 9, 13, 25, 29, 33, 37; Mayhe-Nunes & Brandão, 2007: figs 1, 5, 9, 15, 19, 23; Rabeling *et al.*, 2007: figs 8B, 19B).
2. In frontodorsal view, median pronotal spines absent or strongly reduced; when present, tooth-like and fused at base;

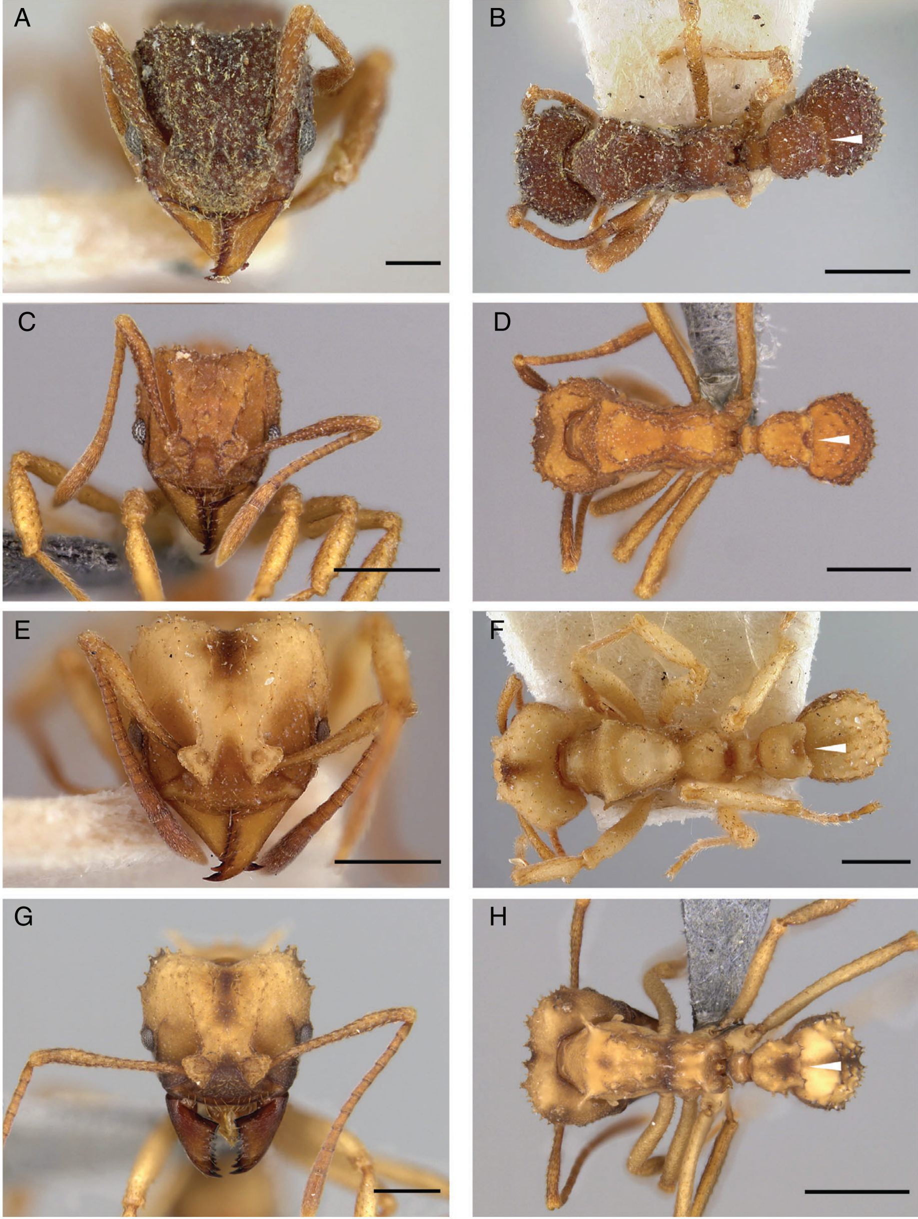

Fig. 4. Species of *Paratrachymyrmex* **gen.n**. (A, B) *Paratrachymyrmex bugnioni* (Forel), type specimen, Forel Collection, Museum d'Histoire Naturelle de Genève, Switzerland (MNGH). (C, D) *Paratrachymyrmex* cf. *carib* (Weber). (E, F) *Paratrachymyrmex cornetzi* (Forel), type specimen, Forel Collection (MNGH). (G, H) *Paratrachymyrmex diversus* (Mann). (I, J) *Paratrachymyrmex intermedius* (Forel), type specimen, Forel Collection (MNGH). (K, L) *Paratrachymyrmex irmgardae* (Forel), type specimen, Forel Collection (MNGH). (A, C, E, G, I, K) Head of workers in full-face view; (B, D, F, H, J, L) workers in dorsal view. White arrows indicate the straight to shallowly concave posterior margin of the postpetiole in dorsal view. Scale bars are 0.5 mm except for (A) (0.2 mm) and (H) (1.0 mm).

in some species apices of both teeth distinguishable, in others, both teeth entirely fused and only recognizable as a single median tooth (Mayhé-Nunes & Brandão, 2005: figs 11, 15, 27, 31, 35, 39; Mayhe-Nunes & Brandão, 2007: figs 2, 7, 11, 17, 21, 25).

3. Dorsal surface of mandible smooth and shiny, without distinct striae; in some species [e.g. *M. turrifex* (Wheeler)] striae present at the base of the mandible.
4. Eyes variable in size and often relatively flat.
5. Widely distributed throughout South America (22 species), with five species in Central America [*M. isthmicus* (Santschi), *M. opulentus* (Mann), *M. squamulifer* (Emery), *M. turrifex* (Wheeler), *M. zeteki* (Weber)], three in the Caribbean [*M. haytianus* (Wheeler and Mann), *M. jamaicensis* (André), *M. jamaicensis antiguensis* (Weber)], and two in North America [*M. jamaicensis* (André), *M. turrifex*

Fig. 4. Continued.

(Wheeler)]. The species are almost evenly divided between seasonally dry habitat (14 species; Cerrado, Restinga, Caatinga, Chaco, desert) and wet forest (13 species).

Species composition: see Table 5.

Etymology: the genus is named in honour of the great mycologist Alfred Möller (1860–1922). During the 3-year period between 1890 and 1893, Möller visited his uncle, the well-known naturalist Fritz Müller, in Blumenau, Brazil, where he undertook pioneering studies of attine fungal cultivars. The resulting monograph, *Die Pilzgärten einiger südamerikanischer Ameisen* (Möller, 1893), remains the seminal work on the subject.

Paratrachymyrmex *Solomon, Rabeling, Sosa-Calvo and Schultz,* ***gen.n.***

Type species: *Paratrachymyrmex intermedius* (Forel, 1909)

Diagnosis:

1. Preocular carina weakly curving mesad above the eye (Fig. 4). In species other than *P. intermedius* (Forel) and *P. diversus* (Mann), the preocular carina is weakly developed.
2. Posterior margin of postpetiole straight, without distinct concave emargination. In some specimens of *P. cornetzi* (Forel), *P. diversus* (Mann), and *P. intermedius* (Forel), the posterior margin is shallowly concave or sinuous (Fig. 4).
3. Eyes variable in size and in most species somewhat flat, barely interrupting the contour of the lateral cephalic margin in full-face view. As far as we know, the only exception is *P. intermedius* (Forel), in which the eyes are convex (Fig. 4).
4. Dorsum of mandibles striate *(P. carib* (Weber), *P. intermedius* (Forel), *P. bugnioni* (Forel), *P. irmgardae* (Forel)) to strigulate [*P. diversus* (Mann), *P. cornetzi* (Forel), *P. levis* (Weber), *P. mandibularis* (Weber)].
5. Species occur mainly in the wet forests of northern South America. *Paratrachymyrmex bugnioni* (Forel), P. *cornetzi* (Forel) and *P. intermedius* (Forel) also occur in Central America.

Species composition: see Table 5.

Etymology: the genus name reflects the fact that this new genus shares many morphological character states with *Trachymyrmex* s.s., making it difficult to separate the genera morphologically.

Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Table S1. Ant and fungal samples analysed for this study, including collector's code (or, if data were taken from GenBank, GenBank accession number prefaced with 'GB'), voucher repository, collection locality, collection latitude and longitude, and collector's name. USNM, Smithsonian Institution National Museum of Natural History, Washington, DC; SIBR, Social Insect Biodiversity Repository, Arizona State University, Tempe, Arizona. GPS coordinates in italic were estimated using Google Earth.

Table S2. Ant gene sequences analysed for this study, including DNA extraction code (DNAex), collector's code

(voucher), and GenBank accession number. GenBank accession numbers in bold were newly generated for this study; missing sequences are indicated by '–'.

Table S3. Fungal gene sequences analysed for this study, including collector's code (voucher) and GenBank accession numbers. GenBank accession numbers in bold were newly generated for this study; missing sequences are indicated by '–'.

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Erratum

In [1], the following errors were published in SYEN 44:4.

The incorrect reference year “Branstetter *et al.*, 1975,” occurred on pages 940, 941, 944, 945, 946, 949, and 950. In all cases, the citation should have read “Branstetter *et al.* 2017.”

The reference, which was erroneously omitted, should have appeared on pp. 953 in the References section and should have read as follows:

‘Branstetter, M.G., Ješovnik, A., Sosa-Calvo, J., Lloyd, M.W., Faircloth, B.C., Brady, S.G. & Schultz, T.R. (2017) Dry habitats were crucibles of domestication in the evolution of agriculture in ants. *Proceedings of the Royal Society of London B*, **284**, 20170095.’

The following reference, which appeared on pp. 953, was out of place and should have been omitted:

‘Gonçalves, C. (1975) Formigas dos gêneros *Sericomyrmex* e *Trachymyrmex* cortando folhas verdes de plantas (Hymenoptera., Formicidae, Myrmicinae, Attini). Reunião Anual da Sociedade Brasileira para o Progresso da Ciência, Universidade Federal de Minas Gerais, Belo Horizonte, Brazil, Vol. 28, p. 1670.’

The reference for Gonçalves 1975 was correctly listed on page 954.

We apologize for these errors.

Reference

1. Solomon, S.E., Rabeling, C., Sosa-Calvo, J. *et al.* (2019) The molecular phylogenetics of *Trachymyrmex* Forel ants and their fungal cultivars provide insights into the origin and coevolutionary history of ‘higher-attine’ ant agriculture. *Systematic Entomology*, **44**, 939–956.

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