

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

SCOTT E. SOLOMON^{1,2}, CHRISTIAN RABELING^{2,3}, JEFFREY SOSA-CALVO^{2,3}, CAUÊ T. LOPES⁴, ANDRÉ RODRIGUES⁵, HERALDO L. VASCONCELOS⁴, MAURÍCIO BACCI Jr⁵, ULRICH G. MUELLER⁶ and TED R. SCHULTZ²

¹Department of BioSciences, Program in Ecology and Evolutionary Biology, Rice University, Houston, TX, U.S.A., ²Department of Entomology, National Museum of Natural History, Smithsonian Institution, Washington, DC, U.S.A., ³School of Life Sciences, Arizona State University, Tempe, AZ, U.S.A., ⁴Instituto de Biologia, Federal University of Uberlândia, Uberlândia, Brazil, ⁵Centro de Estudos de Insetos Sociais, Universidade Estadual Paulista, Rio Claro, Brazil and ⁶Department of Integrative Biology, University of Texas at Austin, Austin, TX, U.S.A.

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

Correspondence: Scott E. Solomon, Department of BioSciences, Program in Ecology and Evolutionary Biology, Rice University, 6100 Main St., MS 170,Houston, TX 77005-1827 U.S.A. E-mail: scott.solomon@rice.edu; Christian Rabeling, School of Life Sciences, Arizona State University, 427 East Tyler Mall. Tempe, AZ 85287-4501 U.S.A. E-mail: christian.rabeling@asu.edu; and Ted R. Schultz, Department of Entomology, National Museum of Natural History, Smithsonian Institution, P.O. Box 37012, Washington, DC, 20013-7012, U.S.A. E-mail: schultzt@si.edu

© 2019 The Authors. *Systematic Entomology* published by John Wiley & Sons Ltd on behalf of Royal Entomological Society. This is an open access article under the terms of the Creative Commons Attribution-NonCommercial-NoDerivs License, which permits use and distribution in any medium, provided the original work is properly cited, the use is non-commercial and no modifications or adaptations are made. 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 *Tra-chymyrmex* 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 $20 \,\mu$ L 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 Ia FI copy, EFIAFI; elongation
factor 1a F2 copy, EF1aF2; long-wavelength rhodopsin, LWRh; 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 <i>et al.</i> (1994)
	HCO2198	TAA ACT TCA GGG TGA CCA AAA AAT CA	Folmer <i>et al.</i> (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 μ L solutions containing 1 μ L of template DNA, 1 μ L of each primer (forward and reverse), 12.2 μ L of H₂O, 5 μ L of 5x buffer, 2 μ L of dNTPs, 2.5 μ L of MgCl₂, and 0.3 μ L of Taq polymerase (Promega, Maddison, WI, U.S.A.) or in 20 μ L solutions containing 1 μ L of template, 0.8 μ L of each primer, 5.4 μ L of H₂O, and 10 μ L of PCR Master Mix (1.5 mM MgCl₂, 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

primers employed in both amplification and sequencing are listed in Table 1. Sequencing reactions used 1 μ L 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 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, Leucoagricus 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

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

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.

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:

- 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.
- 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).

- 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 Gen-Bank, 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 '-'.

Acknowledgements

We are very grateful to the following colleagues for providing samples that were used in this study and/or for facilitating field work: Rachelle Adams, Donat Agosti, Roberto C.F. Brandão, Cameron Currie, Jacques Delabie, Ana Harada, Anna Himler, John Lattke, Inara Leal, Alexandre Marchesi, Antonio Mayhé-Nunes and Eduardo Salomão. We are thankful to Marek Borowiec for sharing his unpublished manuscript to generate the files for submitting sequences to GenBank, and to Eugenia Okonski for creating the images for Fig. 4. This work was supported by the U.S. National Science Foundation (International Research Fellowship Program award 07012333 to SES; DEB-0949689 to TRS: DEB-1456964 and DEB-1654829 to CR and TRS: DEB-0919519 and DEB-1354666 to UGM), the W.M. Wheeler Endowment from the University of Texas at Austin, and Smithsonian Scholarly Studies and CGPS grants to TRS and a Smithsonian Institution Peter Buck Pre-Doctoral Fellowship to JSC. Field work in Brazil was conducted under permit numbers 14789-1, 14789-2, 14789-3, 14789-4, 14789-5, 14789-9, 33487-2 and 33487-4 issued by the Instituto Brasileiro do Meio Ambiente e dos Recursos Naturais Renováveis (IBAMA) and the Instituto Chico Mendes de Conservação da Biodiversidade (ICMBio). The authors state that there are no conflicts of interest.

References

- Abouheif, E. & Wray, G.A. (2002) Evolution of the gene network underlying wing polyphenism in ants. *Science*, **297**, 249–252.
- Albuquerque, E.Z., Diehl-Fleig, E., Diehl, E. & Mayhé-Nunes, A.J. (2018) Sex investment ratios and natural history observations in a population of *Trachymyrmex holmgreni* (Formicidae) in southern Brazil. *Insectes Sociaux*, **65**, 297–303.
- Birnbaum, S.L. & Gerardo, N.M. (2016) Patterns of specificity of the pathogen *Escovopsis* across the fungus-growing ant symbiosis. *The American Naturalist*, 188, 52–65.
- Boucher, D.H. (ed.) (1988) *The Biology of Mutualism: Ecology and Evolution*. Oxford University Press, New York.
- Brady, S.G., Gadau, J. & Ward, P.S. (2000) Systematics of the ant genus *Camponotus* (Hymenoptera: Formicidae): a preliminary analysis using data from the mitochondrial gene Cytochrome Oxidase I. *Hymenoptera: Evolution, Biodiversity, and Biological Control* (ed. by A. Austin and M. Dowton), pp. 131–138. CSIRO Publishing, Collingwood.
- Brady, S.G., Schultz, T.R., Fisher, B.L. & Ward, P.S. (2006) Evaluating alternative hypotheses for the early evolution and diversification of ants. *Proceedings of the National Academy of Sciences of the United States of America*, **103**, 18172–18177.

- Brandão, C.R.F. (1991) Adendos ao catálogo abreviado das formigas da região Neotropical (Hymenoptera: Formicidae). *Revista Brasileira de Biologia*, 35, 319–412.
- Brandão, C.R.F. & Mayhé-Nunes, A.J. (2007) A phylogenetic hypothesis for the *Trachymyrmex* species groups, and the transition from fungus-growing to leaf-cutting in the Attini. *Advances in Ant Systematics (Hymenoptera: Formicidae): Homage to E.O. Wilson*, Memoirs of the American Entomological Institute, **80**, pp. 72–88.
- Gonçalves, C. (1975) Formigas dos géneros Sericomyrmex e Trachymyrmex cortando folhas verdes de plantas (Hymenoptera., Formicidae, Myrmicinae, Attini). *Reunião Anual da Sociedade Brasileira para o Progresso da Ciéncia*, Universidade Federal de Minas Gerais, Belo Horizonte, Brazil, Vol. 28, p. 1670.
- Brown, J.M., Hedtke, S.M., Lemmon, A.R. & Lemmon, E.M. (2010) When trees grow too long: investigating the causes of highly inaccurate Bayesian branch-length estimates. *Systematic Biology*, **59**, 145–161.
- Cafaro, M.J., Poulsen, M., Little, A.E. *et al.* (2011) Specificity in the symbiotic association between fungus-growing ants and protective *Pseudonocardia* bacteria. *Proceedings of the Royal Society of London B*, **278**, 1814–1822.
- Carlson, A.L., Ishak, H.D., Kurian, J., Mikheyev, A.S., Gifford, I. & Mueller, U.G. (2017) Nuclear populations of the multinucleate fungus of leafcutter ants can be dekaryotized and recombined to manipulate growth of nutritive hyphal nodules harvested by the ants. *Mycologia*, 109, 832–846.
- Chapela, I.H., Rehner, S.A., Schultz, T.R. & Mueller, U.G. (1994) Evolutionary history of the symbiosis between fungus-growing ants and their fungi. *Science*, **266**, 1691–1694.
- Cherrett, J.M. (1989) Biology, pest status and control of leaf-cutting ants. *Biology and Population Dynamics of Invertebrate Crop Pests* (ed. by G.E. Russell), pp. 1–38. Intercept, Andover, MN.
- Cristiano, M.P., Cardoso, D.C. & Fernandes-Salomão, T.M. (2013) Cytogenetic and molecular analyses reveal a divergence between *Acromyrmex striatus* (Roger, 1863) and other congeneric species: taxonomic implications. *PLoS One*, **8**, e59784.
- Currie, C.R. (2001) A community of ants, fungi, and bacteria: a multilateral approach to studying symbiosis. *Annual Reviews in Microbiology*, 55, 357–380.
- Currie, C.R., Mueller, U.G. & Malloch, D. (1999a) The agricultural pathology of ant fungus gardens. *Proceedings of the National Academy of Sciences of the United States of America*, **96**, 7998–8002.
- Currie, C.R., Scott, J.A., Summerbell, R.C. & Malloch, D. (1999b) Fungus-growing ants use antibiotic-producing bacteria to control garden parasites. *Nature*, **398**, 701–704.
- Currie, C.R., Wong, B., Stuart, A.E. *et al.* (2003a) Ancient tripartite coevolution in the attine ant-microbe symbiosis. *Science*, **299**, 386–388.
- Currie, C.R., Bot, A.N.M. & Boomsma, J.J. (2003b) Experimental evidence of a tripartite mutualism: bacteria protect ant fungus gardens from specialized parasites. *Oikos*, **101**, 91–102.
- Currie, C.R., Scott, J.A., Summerbell, R.C. & Malloch, D. (2003c) Corrigendum: fungus-growing ants use antibiotic-producing bacteria to control garden parasites. *Nature*, **423**, 461–462.
- Currie, C.R., Poulsen, M., Mendenhall, J., Boomsma, J.J. & Billen, J. (2006) Coevolved crypts and exocrine glands support mutualistic bacteria in fungus-growing ants. *Science*, **311**, 81–83.
- De Fine Licht, H.H. & Boomsma, J.J. (2010) Forage collection, substrate preparation, and diet composition in fungus-growing ants. *Ecological Entomology*, 35, 259–269.
- De Fine Licht, H.H. & Boomsma, J.J. (2014) Variable interaction specificity and symbiont performance in Panamanian *Trachymyrmex* and *Sericomyrmex* fungus-growing ants. *BMC Evolutionary Biology*, **14**, 244.

- De Fine Licht, H.H., Schiøtt, M., Rogowska-Wrzesinska, A., Nygaard, S., Roepstorff, P. & Boomsma, J.J. (2013) Laccase detoxification mediates the nutritional alliance between leaf-cutting ants and fungus-garden symbionts. *Proceedings of the National Academy of Sciences of the United States of America*, **110**, 583–587.
- Della Lucia, T.M.C. (2011) Formigas-Cortadeiras: da Bioecologia ao Manejo Editoria. Universidade Federal de Viçosa, Viçosa.
- Fernández, F. & Sendoya, S. (2004) Synonymic list of Neotropical ants (Hymenoptera: Formicidae). *Biota Colombiana*, 5, 3–93.
- Fernández, F., Castro-Huertas, V. & Serna, F. (2015) Hormigas cortadoras de hojas de Colombia: Acromyrmex & Atta (Hymenoptera: Formicidae). Fauna de Colombia, Monografía No. 5, Instituto de Ciencias Naturales, Universidad Nacional de Colombia, Bogotá D.C., Colombia, 350p.
- Folmer, O., Black, M., Hoeh, W., Lutz, R. & Vrijenhoek, R. (1994) DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. *Molecular Marine Biology and Biotechnology*, **3**, 294–299.
- Fowler, H.G. (1983) Distribution patterns of Paraguayan leaf-cutting ants (*Atta* and *Acromyrmex*) (Formicidae: Attini). *Studies on Neotropical Fauna and Environment*, 18, 121–138.
- Gerardo, N.M., Mueller, U.G., Price, S.L. & Currie, C.R. (2004) Exploiting a mutualism: parasite specialization on cultivars within the fungus-growing ant symbiosis. *Proceedings of the Royal Society of London B*, **271**, 1791–1798.
- Gerardo, N.M., Mueller, U.G. & Currie, C.R. (2006) Complex host-pathogen coevolution in the *Apterostigma* fungus-growing ant-microbe symbiosis. *BioMed Central Evolutionary Biology*, 6, 88.
- 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.
- Gray, K.W., Cover, S.P., Johnson, R.A. & Rabeling, C. (2018) The dacetine ant *Strumigenys arizonica*, an apparent obligate commensal of the fungus-growing ant *Trachymyrmex arizonensis* in southwestern North America. *Insectes Sociaux*, 65, 401–410.
- Heine, D., Holmes, N.A., Worsley, S.F. *et al.* (2018) Chemical warfare between leafcutter ant symbionts and a co-evolved pathogen. *Nature Communications*, 9, 2208.
- Hölldobler, B. & Wilson, E.O. (1990) *The Ants*. Harvard University Press, Cambridge, MA.
- Hölldobler, B. & Wilson, E.O. (2011) *The Leafcutter Ants: Civilization by Instinct*. WW Norton & Company, New York.
- Ješovnik, A. & Schultz, T.R. (2017) Revision of the fungus-farming ant genus *Sericomyrmex* Mayr (Hymenoptera, Formicidae, Myrmicinae). *ZooKeys*, 670, 1–109.
- Ješovnik, A., González, V.L. & Schultz, T.R. (2016) Phylogenomics and divergence dating of fungus-farming ants (Hymenoptera: Formicidae) of the genera Sericomyrmex and Apterostigma. PLoS One, 11, e0151059.
- Ješovnik, A., Sosa-Calvo, J., Lloyd, M.W., Branstetter, M.G., Fernández, F. & Schultz, T.R. (2017) Phylogenomic species delimitation and host-symbiont coevolution in the fungus-farming ant genus *Sericomyrmex* Mayr (Hymenoptera: Formicidae): ultraconserved elements (UCEs) resolve a recent radiation. *Systematic Entomology*, 42, 523–542.
- Katoh, K. & Standley, D.M. (2013) MAFFT multiple sequence alignment software version 7: improvements in performance and usability. *Molecular Biology and Evolution*, **30**, 772–780.
- Katoh, K., Misawa, K., Kuma, K.I. & Miyata, T. (2002) MAFFT: a novel method for rapid multiple sequence alignment based on fast Fourier transform. *Nucleic Acids Research*, **30**, 3059–3066.

- Katoh, K., Kuma, K.I., Toh, H. & Miyata, T. (2005) MAFFT version 5: improvement in accuracy of multiple sequence alignment. *Nucleic Acids Research*, 33, 511–518.
- Kearse, M., Moir, R., Wilson, A. *et al.* (2012) Geneious Basic: an integrated and extendable desktop software platform for the organization and analysis of sequence data. *Bioinformatics*, 28, 1647–1649.
- Kempf, W.W. (1972) Catálogo abreviado das formigas da região Neotropical. *Studia Entomologica*, 15, 3–344.
- Kooij, P.W., Aanen, D.K., Schiøtt, M. & Boomsma, J.J. (2015) Evolutionarily advanced ant farmers rear polyploid fungal crops. *Journal of Evolutionary Biology*, 28, 1911–1924.
- Kusnezov, N. (1963) Zoogeografía de las hormigas en Sudamérica. Acta Zoológica Lilloana, **19**, 25–186.
- Lanfear, R., Calcott, B., Ho, S.Y.W. & Guindon, S. (2012) Partition-Finder: combined selection of partitioning schemes and substitution models for phylogenetic analyses. *Molecular Biology and Evolution*, 29, 1695–1701.
- Li, H., Sosa-Calvo, J., Horn, H.A. et al. (2018) Convergent evolution of complex structures for ant-bacterial defensive symbiosis in fungus-farming ants. Proceedings of the National Academy of Sciences of the United States of America, 115, 10720–10725.
- Lizidatti, C.S. (2006) Biologia, arquitetura de ninhos e coleta de substratos no cerrado por formigas cultivadoras de fungo, Trachymyrmex holmgreni Wheeler, 1925 (Hymenoptera, Formicidae, Attini). Master Thesis, UNESP, Rio Claro.
- Maddison, W.P. & Maddison, D.R. (2015). *Mesquite: A Modular System for Evolutionary Analysis, Version 3.0.* URL http://mesquiteproject.org [accessed on 1 July 2018].
- Mann, WM. (1916). The ants of Brazil. Bulletin of the Museum of Comparative Zoology of Harvard College, 60, 399–490.
- Marshall, D.C. (2010) Cryptic failure of partitioned Bayesian phylogenetic analyses: lost in the land of long trees. *Systematic Biology*, 59, 108–117.
- Marshall, D.C., Simon, C. & Buckley, T.R. (2006) Accurate branch length estimation in partitioned Bayesian analyses requires accommodation of among-partition rate variation and attention to branch length priors. *Systematic Biology*, **55**, 993–1003.
- Masiulionis, V.E., Rabeling, C., De Fine Licht, H.H., Schultz, T.R., Bacci, M. Jr, Bezerra, C.M.S. & Pagnocca, F.C. (2014) A Brazilian population of the asexual fungus-growing ant *Mycocepurus smithii* (Formicidae, Myrmicinae, Attini) cultivates fungal symbionts with gongylidia-like structures. *PLoS One*, 9, e103800.
- Mayhé-Nunes, A.J. & Brandão, C.R.F. (2002) Revisionary studies on the attine ant genus *Trachymyrmex* Forel. Part 1: definition of the genus and the *Opulentus* group (Hymenoptera: Formicidae). *Sociobiology*, 40, 667–698.
- Mayhé-Nunes, A.J. & Brandão, C.R.F. (2005) Revisionary studies on the attine ant genus *Trachymyrmex* Forel. Part 2: the *Iheringi* group (Hymenoptera: Formicidae). *Sociobiology*, **45**, 271–305.
- Mayhe-Nunes, A.J. & Brandão, C.R.F. (2007) Revisionary studies on the attine ant genus *Trachymyrmex* Forel. Part 3: the *Jamaicensis* group (Hymenoptera: Formicidae). *Zootaxa*, **1444**, 1–21.
- Mayhé-Nunes, A.J. & Jaffé, K. (1998) On the biogeography of Attini (Hymenoptera: Formicidae). *Ecotropicos*, 11, 45–54.
- Mehdiabadi, N.J. & Schultz, T.R. (2010) Natural history and phylogeny of the fungus-farming ants (Hymenoptera: Formicidae: Myrmicinae: Attini). *Myrmecological News*, **13**, 37–55.
- Mehdiabadi, N.J., Mueller, U.G., Brady, S.G., Himler, A.G. & Schultz, T.R. (2012) Symbiont fidelity and the origin of species in fungus-growing ants. *Nature Communications*, 3, 840–846.
- Meirelles, L.A., Mendes, T.D., Solomon, S.E., Bueno, O.C., Pagnocca, F.C. & Rodrigues, A. (2014) Broad *Escovopsis*-inhibition activity of *Pseudonocardia* associated with *Trachymyrmex* ants. *Environmental Microbiology Reports*, 6, 339–345.

- Meirelles, L.A., Montoya, Q.V., Solomon, S.E. & Rodrigues, A. (2015) New light on the systematics of fungi associated with attine ant gardens and the description of *Escovopsis kreiselii* sp. nov. *PLoS One*, **10**, e0112067.
- Mikheyev AS, Mueller UG, & Abbot P (2006) Cryptic sex and many-to-one coevolution in the fungus-growing ant symbiosis. *Pro*ceedings of the National Academy of Sciences 103, 10702–10706.
- Mikheyev, A.S., Mueller, U.G. & Boomsma, J.J. (2007) Population genetic signatures of diffuse co-evolution between leaf-cutting ants and their cultivar fungi. *Molecular Ecology*, 16, 209–216.
- Mikheyev, A.S., Mueller, U.G. & Abbot, P. (2010) Comparative dating of attine ant and lepiotaceous cultivar phylogenies reveals coevolutionary synchrony and discord. *The American Naturalist*, **175**, E126–E133.
- Möller, A. (1893) Die Pilzgärten einiger südamerikanischer Ameisen. Botanische Mittheilungen aus den Tropen, Jena, Germany, Vol. 6, pp. 1–127.
- Mueller, U.G. (2002) Ant versus fungus versus mutualism: ant-cultivar conflict and the deconstruction of the attine ant-fungus symbiosis. *The American Naturalist*, 160, S67–S98.
- Mueller, U.G. (2012) Symbiont recruitment versus ant-symbiont co-evolution in the attine ant-microbe symbiosis. *Current Opinion* in Microbiology, 15, 269–277.
- Mueller, U.G. & Rabeling, C. (2008) A breakthrough in animal evolution. Proceedings of the National Academy of Sciences of the United States of America, 105, 5287–5288.
- Mueller, U.G., Rehner, S.A. & Schultz, T.R. (1998) The evolution of agriculture in ants. *Science*, 281, 2034–2038.
- Mueller, U.G., Schultz, T.R., Currie, C.R., Adams, R.M.M. & Malloch, D. (2001) The origin of the attine ant-fungus mutualism. *Quarterly Review of Biology*, **76**, 169–197.
- Mueller, U.G., Gerardo, N.M., Aanen, D.K., Six, D.L. & Schultz, T.R. (2005) The evolution of agriculture in insects. *Annual Review of Ecology, Evolution, and Systematics*, 36, 563–595.
- Mueller, U.G., Dash, D., Rabeling, C. & Rodrigues, A. (2008) Coevolution between attine ants and Actinomycete bacteria: a reevaluation. *Evolution*, **62**, 2894–2912.
- Mueller, U.G., Ishak, H.D., Bruschi, S.M. *et al.* (2017) Biogeography of mutualistic fungi cultivated by leafcutter ants. *Molecular Ecology*, 26, 6921–6937.
- Mueller, U.G., Kardish, M.R., Ishak, H.D. *et al.* (2018) Phylogenetic patterns of ant–fungus associations indicate that farming strategies, not only a superior fungal cultivar, explain the ecological success of leafcutter ants. *Molecular Ecology*, 27, 2414–2434.
- Murakami, T., Higashi, S. & Windsor, D. (2000) Mating frequency, colony size, polyethism and sex ratio in fungus-growing ants (Attini). *Behavioral Ecology and Sociobiology*, 48, 276–284.
- Newton, M.A. & Raftery, A.E. (1994) Approximate Bayesian inference with the weighted likelihood bootstrap. *Journal of the Royal Statisti*cal Society Series B (Methodological), 56, 3–48.
- Nygaard, S., Hu, H., Li, C. *et al.* (2016) Reciprocal genomic evolution in the ant–fungus agricultural symbiosis. *Nature Communications*, **7**, 12233.
- O'Dea, A., Lessios, H.A., Coates, A.G. *et al.* (2016) Formation of the isthmus of Panama. *Science Advances*, **2**, e1600883.
- Oster, G.F. & Wilson, E.O. (1979) *Caste and Ecology in the Social Insects*. Princeton University Press, Princeton, NJ.
- Rabeling, C., Cover, S.P., Johnson, R.A. & Mueller, U.G. (2007a) A review of the North American species of the fungus-gardening ant genus *Trachymyrmex* (Hymenoptera: Formicidae). *Zootaxa*, **1664**, 1–53.
- Rabeling, C., Verhaagh, M. & Engels, W. (2007b) Comparative study of nest architecture and colony structure of the fungus-growing ants,

Mycocepurus goeldii and M. smithii. Journal of Insect Science, 7, 1–13.

- Rabeling, C., Brown, J.M. & Verhaagh, M. (2008) Newly discovered sister lineage sheds light on early ant evolution. *Proceedings of the National Academy of Sciences of the United States of America*, **105**, 14913–14917.
- Rabeling, C., Schultz, T.R., Bacci, M. Jr & Bollazzi, M. (2015) Acromyrmex charruanus: a new inquiline social parasite species of leaf-cutting ants. Insectes Sociaux, 62, 335–349.
- Rambaut, A., Drummond, A.J., Xie, D., Baele, G. & Suchard, M.A. (2018) Posterior summarization in Bayesian phylogenetics using Tracer 1.7. *Systematic Biology*, **67**, 901–904.
- Ronquist, F., Teslenko, M., van der Mark, P. et al. (2012) MrBayes 3.2: efficient Bayesian phylogenetic inference and model choice across a large model space. Systematic Biology, 61, 539–542.
- Sánchez-Peña, S.R., Chacón-Cardosa, M.C., Canales-del-Castillo, R., Ward, L. & Resendez-Pérez, D. (2017) A new species of *Tra-chymyrmex* (Hymenoptera, Formicidae) fungus-growing ant from the Sierra Madre Oriental of northeastern Mexico. *ZooKeys*, **706**, 73–94.
- Scheuring, I. & Yu, D.W. (2012) How to assemble a beneficial microbiome in three easy steps. *Ecology Letters*, 15, 1300–1307.
- Schultz, T.R. & Brady, S.G. (2008) Major evolutionary transitions in ant agriculture. Proceedings of the National Academy of Sciences of the United States of America, 105, 5435–5440.
- Schultz, T.R. & Meier, R. (1995) A phylogenetic analysis of the fungus-growing ants (Hymenoptera: Formicidae: Attini) based on morphological characters of the larvae. *Systematic Entomology*, 20, 337–370.
- Schultz, T.R., Mueller, U.G., Currie, C.R. & Rehner, S.A. (2005) Reciprocal illumination. A comparison of agriculture in humans and in fungus–growing ants. *Insect–Fungal Associations: Ecology and Evolution* (ed. by F.E. Vega and M. Blackwell), pp. 149–190. Oxford University Press, New York.
- Schultz, T.R., Sosa-Calvo, J., Brady, S.G., Lopes, C.T., Mueller, U.G., Bacci, M. Jr & Vasconcelos, H.L. (2015) The most relictual fungus-farming ant species cultivates the most recently evolved and highly domesticated fungal symbiont species. *The American Naturalist*, **185**, 693–703.
- Scott, J.J., Kweskin, M., Cooper, M. & Mueller, U.G. (2009) Polymorphic microsatellite markers for the symbiotic fungi cultivated by leaf cutter ants (Attini, Formicidae). *Molecular Ecology Resources*, 9, 1391–1394.
- Seal, J.N. & Mueller, U.G. (2014) Instability of novel ant-fungal associations constrains horizontal exchange of fungal symbionts. *Evolutionary Ecology*, 28, 157–176.
- Seal, J.N. & Tschinkel, W.R. (2006) Colony productivity of the fungus-gardening ant *Trachymyrmex septentrionalis* (Hymenoptera: Formicidae) in a Florida pine forest. *Annals of the Entomological Society of America*, **99**, 673–682.
- Seal, J.N. & Tschinkel, W.R. (2007a) Co-evolution and the superorganism: switching cultivars does not alter the performance of fungus-gardening ant colonies. *Functional Ecology*, 21, 988–997.
- Seal, J.N. & Tschinkel, W.R. (2007b) Energetics of newly-mated queens and colony founding in the fungus-gardening ants *Cyphomyrmex rimosus* and *Trachymyrmex septentrionalis* (Hymenoptera : Formicidae). *Physiological Entomology*, **32**, 8–15.
- Seal, J.N. & Tschinkel, W.R. (2007c) Complexity in an obligate mutualism: do fungus-gardening ants know what makes their garden grow? *Behavioral Ecology and Sociobiology*, 61, 1151–1160.
- Seal, J.N. & Tschinkel, W.R. (2008) Food limitation in the fungus-gardening ant, *Trachymyrmex septentrionalis*. *Ecological Entomology*, 33, 597–607.
- © 2019 The Authors. Systematic Entomology published by John Wiley & Sons Ltd on behalf of Royal Entomological Society. 44, 939–956

- Seal, J.N., Schiøtt, M. & Mueller, U.G. (2014) Ant-fungus species combinations engineer physiological activity of fungus gardens. *Journal of Experimental Biology*, 217, 2540–2547.
- Simon, C., Frati, F., Beckenbach, A., Crespi, B., Liu, H. & Flook, P. (1994) Evolution, weighting, and phylogenetic utility of mitochondrial gene sequences and a compilation of conserved polymerase chain reaction primers. *Annals of the Entomological Society of America*, 87, 651–701.
- Solomon, S.E., Lopes, C.T., Mueller, U.G., Rodrigues, A., Sosa-Calvo, J., Schultz, T.R. & Vasconcelos, H.L. (2011) Nesting biology and fungiculture of the fungus-growing ant, *Mycetagroicus cerradensis*: new light on the origin of higher attine agriculture. *Journal of Insect Science*, **11**, 1–14.
- Sosa-Calvo, J., Schultz, T.R., Brandão, C.R. et al. (2013) Cyatta abscondita: taxonomy, evolution, and natural history of a new fungus-farming ant genus from Brazil. PLoS One, 8, e80498.
- Sosa-Calvo, J., Jesovnik, A., Okonski, E. & Schultz, T.R. (2015) Locating, collecting, and maintaining colonies of fungus-farming ants (Hymenoptera: Myrmicinae: Attini). *Sociobiology*, **62**, 300–320.
- Sosa-Calvo, J., Schultz, T.R., Ješovnik, A., Dahan, R. & Rabeling, C. (2018) Evolution, systematics, and natural history of a new genus of cryptobiotic fungus-growing ants. *Systematic Entomology*, **43**, 549–567.
- Stamatakis, A. (2006) RAxML-VI-HPC: maximum likelihood-based phylogenetic analyses with thousands of taxa and mixed models. *Bioinformatics*, 22, 2688–2690.
- Suchard, M.A., Weiss, R.E. & Sinsheimer, J.S. (2001) Bayesian selection of continuous-time Markov chain evolutionary models. *Molecular Biology and Evolution*, **18**, 1001–1013.
- Urich, F.W. (1895) Notes on some fungus-growing ants of Trinidad. Journal of the Trinidad Field Naturalists' Club, 2, 175–182.

- Vilgalys, R. & Hester, M. (1990) Rapid genetic identification and mapping of enzymatically amplified ribosomal DNA from several *Cryptococcus* species. *Journal of Bacteriology*, **172**, 4238–4246.
- Villesen, P., Gertsch, P.J., Frydenberg, J., Mueller, U.G. & Boomsma, J.J. (1999) Evolutionary transition from single to multiple mating in fungus-growing ants. *Molecular Ecology*, 8, 1819–1825.
- Villesen, P., Murakami, T. & Schultz, T.R. (2002) Identifying the transition between single and multiple mating of queens in fungus-growing ants. *Proceedings of the Royal Society of London B*, 269, 1541–1548.
- Vo, T.L., Mueller, U.G. & Mikheyev, A.S. (2009) Free-living fungal symbionts (Lepiotaceae) of fungus-growing ants (Attini: Formicidae). *Mycologia*, **101**, 206–210.
- Ward, P. & Downie, D. (2005) The ant subfamily Pseudomyrmecinae (Hymenoptera : Formicidae): phylogeny and evolution of big-eyed arboreal ants. *Systematic Entomology*, **30**, 310–335.
- Weber, N.A. (1972) Gardening Ants, the Attines. The American Philosophical Society, Philadelphia, PA.
- Wheeler, W.M. (1907) The fungus-growing ants of North America. Bulletin of the American Museum of Natural History, 23, 669–807.
- Wheeler, W.M. (1911) Two fungus-growing ants from Arizona. *Psyche*, **18**, 93–101.
- White, T.J., Bruns, T., Lee, S.J.W.T. & Taylor, J.W. (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. *PCR Protocols: A Guide to Methods and Applications* (ed. by M.A. Innis, D.H. Gelfand, J.J. Sninsky and T.J. White), pp. 315–322. Academic Press, Inc., New York, New York.
- Wilson, E.O. (1971) The Insect Societies. Belknap, Cambridge, MA.

Accepted 17 April 2019