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Phylogenomic species delimitation and host-symbiont coevolution in the fungus-farming ant genus *Sericomyrmex* Mayr (Hymenoptera: Formicidae): ultraconserved elements (UCEs) resolve a recent radiation

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Abstract. Ants in the Neotropical genus *Sericomyrmex* Mayr cultivate fungi for food. Both ants and fungi are obligate, coevolved symbionts. The taxonomy of *Sericomyrmex* is problematic because the morphology of the worker caste is generally homogeneous across all of the species within the genus, species limits are vague, and the relationships between them are unknown. We used ultraconserved elements (UCEs) as genome-scale markers to reconstruct evolutionary history and to infer species boundaries in *Sericomyrmex*. We recovered an average of ~990 UCE loci for 88 *Sericomyrmex* samples from across the geographical range of the genus as well as for five outgroup taxa. Using maximum likelihood and species-tree approaches, we recovered nearly identical topologies across datasets with 50–95% matrix completeness. We identify nine species-level lineages in *Sericomyrmex*, including two new species. This is less than the previously described 19 species, even accounting for two species for which we had no UCE samples, which brings the total number of *Sericomyrmex* species to 11. Divergence-dating analyses recovered 4.3 Ma as the crown-group age estimates for *Sericomyrmex,* indicating a recent, rapid radiation. We also sequenced mitochondrial cytochrome oxidase subunit I (COI) for 125 specimens. Resolution and support for clades in our COI phylogeny are weak, indicating that COI is not an appropriate species-delimitation tool. However, taxa within species consistently cluster together, suggesting that COI is useful as a species identification ('DNA barcoding') tool. We also sequenced internal transcribed spacer (ITS) and large subunit (LSU) for 32 *Sericomyrmex* fungal cultivars. The fungal phylogeny confirms that *Sericomyrmex* fungi are generalized higher-attine cultivars, interspersed with *Trachymyrmex*-associated fungal species, indicating cultivar sharing and horizontal transfer between these two genera. Our results indicate that UCEs offer immense potential for delimiting and resolving relationships of problematic, recently diverged species.

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Introduction

The practice of alpha taxonomy, defining and delimiting species, has evolved considerably over the last two decades due to the rise of multidisciplinary species delimitation (Sites & Marshall, 2003; Schlick-Steiner *et al.*, 2010; Yeates *et al.*, 2011). This integrative approach to taxonomy advocates the combination of evidence from several different sources: molecular, ecological, chemical and/or behavioural, in addition to traditional morphological characters, especially for what are considered difficult groups (Martin *et al.*, 2008; Ross *et al.*, 2010; Seppa *et al.*, 2011; Ward, 2011; Ronque *et al.*, 2016). In ants, integrative taxonomy is especially useful in cases where various processes obscure species boundaries, e.g. hybridization (Helms Cahan & Vinson, 2003; Anderson *et al.*, 2006; Steiner *et al.*, 2011; Kulmuni & Pamilo, 2014), morphologically cryptic species (Schlick-Steiner *et al.*, 2006), high interspecific variability (Blaimer & Fisher, 2013; Branstetter, 2013), rapid or recent divergences (Goropashnaya *et al.*, 2004) and clonal reproduction (Pearcy *et al.*, 2004; Foucaud *et al.*, 2010; Rabeling *et al.*, 2011). Most recently, advances in DNA sequencing technology have made genomic data affordable, effecting major changes in our ability to reconstruct deeper evolutionary divergences, such as relationships between families or genera (Johnson *et al.*, 2013; Blaimer *et al.*, 2015), and to resolve species boundaries (Wang *et al.*, 2011; Crawford *et al.*, 2015; Manthey *et al.*, 2016), especially when other approaches fail.

The genus *Sericomyrmex* Mayr is one such problematic ant taxon (Wheeler, 1925; Ješovnik *et al.*, 2016). It belongs to the fungus-farming ants (Formicidae: Myrmicinae: Attini: *Atta* genus group, hereafter referred to as attine ants), a New World clade comprising over 240 extant species that depend on the fungi they farm for food (Schultz & Brady, 2008), and whose most well-known representatives, leaf-cutting ants, are major herbivores in the Neotropics (Wirth & Leal, 2007). Attine ants, and leaf-cutters in particular, have become model organisms for the study of symbiosis and coevolution, and due to years of research, attine ants are better understood than many other comparable ant groups (Mehdiabadi & Schultz, 2010; Nygaard *et al.*, 2016). Fungus-farming ants in the genus *Sericomyrmex*, however, have received little attention, and our knowledge of their evolution, taxonomy, natural history and fungal cultivars remains poor. With 19 described species and three subspecies, *Sericomyrmex* has radiated into a diverse biota across a large geographic area, occupying wet and dry forests, pastures, cerrado, and urban and agricultural habitats from southern Brazil to northern Mexico, and from sea level to over 1000 m in elevation (Mayhe-Nunes & Jaffe, 1998; Fernández & Sendoya, 2004; Bolton, 2014). Surprisingly, this species-level diversity and geographic coverage are accompanied by remarkably little morphological variation. The genus itself is highly distinctive and unmistakenly diagnosable from other attine genera, but species-level variation is subtle and complex. S*ericomyrmex* ants are small- to medium-sized, brown and covered with a dense layer of hairs, giving them a velvety appearance. Differences between most species are subtle and are obscured by sometimes marked intraspecific and intracolonial variation, as some species are mildly polymorphic (Wheeler, 1925; Weber, 1972). A comprehensive taxonomic revision has never been attempted, and our knowledge of species boundaries still relies on the literature from the beginning of the 20th Century (Wheeler, 1916). A recent genomic study that included three *Sericomyrmex* transcriptomes found strong evidence that *Sericomyrmex* species are probably the product of a very recent radiation and that they are separated from their nearest relatives, the *Trachymyrmex iheringi* clade, by a long branch (Ješovnik *et al.*, 2016). However, a more recent molecular phylogeny of the attine ants reveals that the sister of *Sericomyrmex* is *Mycetosoritis explicatus* Kempf (M.G. Branstetter *et al.*, unpublished data), an enigmatic species considered by some authors to be a lower attine (Forel, 1912; Kempf, 1968; Weber, 1972), and by others as a higher attine (Sosa-Calvo *et al.*, 2009).

Because variations in both morphology and nuclear gene sequence data (Ješovnik *et al.*, 2016) are very low across *Sericomyrmex* species – probably the result of a recent, rapid radiation – we consider *Sericomyrmex* to be a good model for judging the utility of a phylogenomic approach for delimiting closely related, recently diverged species. We chose ultraconserved elements (UCEs) as our phylogenomic markers. UCE loci are characterized by having a conserved core and increasingly variable flanking regions (Bejerano *et al.*, 2004). This gradient of variation makes UCEs useful for recovering phylogenetic relationships ranging from deep to shallow scales (McCormack *et al.*, 2012; Smith *et al.*, 2014), including population-level studies and studies of rapid evolutionary radiations (Lim & Braun, 2015; Meiklejohn *et al.*, 2016). Recently developed UCE probes for Hymenoptera (Faircloth *et al.*, 2015) have been used successfully in multiple phylogenetic studies of ants (Blaimer *et al.*, 2015, 2016a). Here we use UCEs to reconstruct the evolutionary history of *Sericomyrmex* and estimate its divergence times, with five outgroup and 88 ingroup samples from across the geographical range of the genus (Fig. 1). Among other purposes, this phylogeny serves as the foundation for a species-level taxonomic revision of *Sericomyrmex* (A. Ješovnik & T.R. Schultz, unpublished data). In addition to UCEs, we sequenced two fragments of the mitochondrial cytochrome oxidase subunit I gene (COI) for most UCE samples plus an additional 38 *Sericomyrmex* specimens, and we generated a COI phylogeny, which we compare with the results of analyses of the UCE data. Although UCEs and similar methods are rapidly becoming easier and cheaper to use, they are still expensive relative to Sanger sequencing techniques. This is especially true for researchers in developing countries where *Sericomyrmex* occurs, including those conducting ecological and conservation studies. Despite its well-known shortcomings (Brower, 2006; Rubinoff *et al.*, 2006), COI has proven useful as a tool for species identification in some ant groups (Smith *et al.*, 2005; Ng'endo *et al.*, 2013). By comparing our UCE and COI results, we can judge the usefulness of COI for species identifications in *Sericomyrmex*.

Finally, to examine host–symbiont coevolution in *Sericomyrmex*, we obtained DNA sequences for the fungal cultivars of *Sericomyrmex* ant species. Attine ants have been obligately dependent on their fungi since soon after their origin around 55–60 million years ago (Ma) (Schultz & Brady, 2008; Ward

Fig. 1. Collection localities. Localities from which *Sericomyrmex* ants (white circles) and fungi (black triangles) were collected. [Colour figure can be viewed at wileyonlinelibrary.com].

et al., 2015, M.G. Branstetter *et al*., unpublished data), whereas, so far as is known, most (but not all) attine fungal species (phylum Basidiomycota, order Agaricales) are facultative symbionts (Ješovnik *et al.*, 2016; Nygaard *et al.*, 2016) capable of living freely outside the attine symbiosis. The cultivation of facultatively symbiotic fungi, practised by the majority of attine genera, is known as 'lower' attine agriculture (Chapela *et al.*, 1994; Hinkle *et al.*, 1994; Mueller *et al.*, 1998; Vo *et al.*, 2009). In contrast, the so-called 'higher' attine fungi, which originated from a lower-attine fungal ancestor, form a clade cultivated by so-called 'higher' attine ants (with one notable exception; see Schultz *et al.*, 2015). Higher-attine fungi are obligate symbionts, incapable of living apart from the ants, and they have evolved various adaptations for life with ants (Schultz & Brady, 2008; Mehdiabadi & Schultz, 2010; Masiulionis *et al.*, 2014; Nygaard *et al.*, 2016).

Sericomyrmex cultivars are higher-attine fungi. Previous phylogenetic studies of attine fungi have included a few *Sericomyrmex* fungal cultivars (De Fine Licht *et al.*, 2010; De Fine Licht & Boomsma, 2014; Kooij *et al.*, 2015), but no prior phylogeny has included fungi from multiple *Sericomyrmex* species spanning the geographical range of the genus, as is done here. Our investigation of *Sericomyrmex* fungi was guided by two aims. First, we wanted to determine whether the radiation of *Sericomyrmex* ant species was driven by coevolution with a specialized clade of higher-attine fungi, as has been suggested for *Atta* (Schultz & Brady, 2008; Mueller *et al.*, 2010). Second, we wanted to quantify the degree to which *Sericomyrmex* ants and

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fungi have coevolved. In particular, we wanted to investigate the degree of ant–fungus symbiont fidelity across the genus or in any subset of species pairs. Even though general patterns of attine ant–fungus coevolution have been described as diffuse coevolution (Mikheyev *et al.*, 2007), it is also the case that phylogenies of attine ants and their fungal cultivars are strongly congruent at deeper phylogenetic levels (Schultz *et al.*, 2015), and that ant and fungal associations in the *Cyphomyrmex wheeleri* species group conform to a pattern of strong symbiont fidelity (Mehdiabadi *et al.*, 2012).

Materials and methods

Field work

Field work was conducted in Guyana, Peru, Mexico, Guatemala, and in multiple localities in Brazil. In addition to those collected in the field, samples were obtained from colleagues and museum collections. Figure 1 illustrates collecting localities for all *Sericomyrmex* ant and fungal specimens used in this study. Table S1 lists all ant specimens used in this study, including collecting information, voucher numbers and GenBank accession numbers. Voucher specimens are deposited in the Smithsonian Institution's National Museum of Natural History in Washington (NMNH), DC, with all data publicly available online in the Museum's database (http://collections.nmnh.si.edu/search/ento) and on AntWeb (http://www.antweb.org). Table S2 contains the same information for fungal specimens. Methods for locating and excavating *Sericomyrmex* nests in the field follow Sosa-Calvo *et al.* (2015). After opening a nest chamber in the field, we used flame-sterilized soft metal forceps to immediately transfer fungus from the fungus garden into vials of 95% ethanol. Field-collected ant samples were likewise preserved in 95% ethanol. Alcohol-preserved material borrowed from museums and colleagues was preserved in ethanol of various concentrations. The map for Fig. 1 was prepared with ARCGIS v10.3.1 software (Esri, Redlands, CA) using a basemap from the U.S. National Park Service.

Taxon selection

Specimens for UCE sequencing were selected to encompass the full range of morphological and molecular variation, as well as geographical distribution, of *Sericomyrmex*. Molecular variation was preliminarily assessed based on two fragments of mitochondrial COI (885 base pairs) sequenced for 125 specimens. Outgroup taxa were chosen based on a recent attine phylogenomic study (M.G. Branstetter *et al*., unpublished data). These included the newly identified sister species of *Sericomyrmex*, *M. explicatus*, and four species from the *Trachymyrmex iheringi* clade, the large sister clade of *Sericomyrmex* + *M. explicatus*. Two *Sericomyrmex* species were available only as old, point-mounted, museum specimens: *Sericomyrmex lutzi*, for which, due to the small number of specimens, we did not attempt any DNA extractions, and *Sericomyrmex* n. sp. 3 from Venezuela, for which we only sequenced COI.

Laboratory methods for Sanger sequencing

We conducted all laboratory work in the Laboratories of Analytical Biology (LAB) at the National Museum of Natural History (NMNH), Smithsonian Institution, in Washington, D.C., U.S.A.

Ants. For the COI dataset we assembled 135 specimens of *Sericomyrmex*. We extracted ants destructively or non-destructively (for rare specimens) using the DNeasy Blood and Tissue Kit (Qiagen, Valencia, CA, U.S.A.) and following the standard protocol described by the manufacturer, except that we prolonged the tissue lysis step (incubation with proteinase K) to overnight. For the final DNA elution step we used two washes of 50 μL double-distilled water (ddH_2O) each, ending with 100 μL of genomic DNA. Amplification for two fragments of COI, defined by primer pairs CI13–CI14 (~560 bp) and Ben-Jerry (~380 bp), was attempted for all specimens with PCR Master Mix (Go Taq and Hot Start kits; Promega, Madison, WI). We ran 15 μL PCR reactions with 1 μL of DNA extract with several different protocols (Table S3), but mostly with a 48°C annealing temperature. Out of 135 extractions performed, ten failed to amplify or sequence due to DNA degradation, and thus the COI dataset contained 125 newly sequenced taxa.

Fungi. We assembled 38 samples of *Sericomyrmex* fungal cultivars, each collected from a different *Sericomyrmex* nest, and attempted to sequence two nuclear ribosomal regions: internal transcribed spacer (ITS) and large subunit (LSU). For each sample we took a small amount of ethanol-preserved fungal tissue, dried it for 1–3 min on tissue paper, and placed it in a 2.0 mL tube that was previously partly filled with 2.3 mm zirconia/silica beads and 0.5 mm glass beads (BioSpec Products, Bartlesville, OK, U.S.A.). We submerged the tubes in liquid nitrogen for 3 min, and then put them into the TissueLyser (Qiagen) for 1 min to ensure tissue disruption. After tissue disruption we extracted DNA from each sample manually using the Plant DNeasy kit (Qiagen) following the standard manufacturer protocol. For the final elutions we used 100 μL of elution buffer. We quantified DNA concentrations for all extracts with a Qubit 2.0 fluorometer high sensitivity kit (Life Technologies, Rockville, MD, U.S.A.). Because fungal samples with high DNA concentrations can be problematic to amplify, we made a series of dilutions for all the samples, resulting in increased amplification success. We prepared the first dilution by mixing 15 μL of the DNA extract with 15 μL of ddH_2O (1:1 dilution); each subsequent dilution was prepared by diluting 15 μL of the previous dilution with another 15 μL of ddH_2O. This was repeated three times for a final dilution of 12.5% of the original extraction. Based on our PCR results, the optimal DNA concentration for PCR amplification was between 0.01 and 0.1 ng/μL. For PCR reactions we used a PCR Master Mix (Hot Start kit). For samples that repeatedly did not amplify, we ran 10 μL of DNA extract on a low-concentration agarose gel (60 min, 60 V) to check for DNA fragmentation. Four of the fungal samples repeatedly failed to PCR-amplify due to degraded DNA. If the

DNA did not seem severely fragmented, we purified it with a generic SPRI substitute Sera-Mag Magnetic SpeedBeads (GE Healthcare, Chicago, IL, U.S.A.), hereafter 'speedbeads' (Fisher *et al.*, 2011; Rohland & Reich, 2012), to remove possible PCR inhibitors (see File S1 for detailed protocol).

In addition to fungal DNA samples, we tried to amplify fungal DNA from ant DNA extractions, a method that was partially successful in a previous study of lower-attine ant genera (Sosa-Calvo, 2015). We ran PCR reactions with fungal primers on 64 samples of destructively extracted ant DNA. We tried the following primer combinations for both fungal and ant extracts: long ITS (ITS1F-LR1, ITS5-ITS4), ITS fragment 1 (ITS1F-ITS2, ITS1-ITS2, ITS1-5.8S), ITS fragment 2 (ITS3-ITS4, ITS4-LR1), LSU (ITS3-LR16, LR3R-LR7, LROR-LR3-1) and SSU (BMB-BR-ITS2). (Table S3).

We succeeded in amplifying fungal DNA from six out of 64 ant extractions. For two of those six ants we had also sampled and sequenced the fungus garden, which enabled us to confirm that the fungus sequenced from the ant DNA extract was identical to the fungus grown in the nest. We sequenced a total of 32 different fungi from ants and fungi combined.

Laboratory methods for UCEs

Extraction and library preparation. We destructively extracted ants as described earlier, except for a few modifications. For the final DNA elution step, we used 130 μL of ddH_2O, to ensure that we had enough extract for all of the quality checks and library preparation. We quantified the DNA extracts with the Qubit 2.0 fluorometer broad range kit (Life Technologies) and ran 10 μL of each extract on an agarose gel to check for possible DNA fragmentation. We sheared 61–417 ng of DNA extract to an average fragment length of 500–600 bp with a Qsonica Q800r sonicator (Qsonica LLC, Newton, CT, U.S.A.) and then used the sheared DNA for library preparation. Library preparations were carried out in 1.5 mL tubes. For 22 *Sericomyrmex* and all five outgroup samples we used the standard Kapa library preparation kit (Faircloth *et al.*, 2015). For the remaining 66 *Sericomyrmex* samples, sequenced subsequently, we used a Kapa Hyper Prep Library Kit (Kapa Biosystems, Wilmington, MA, U.S.A.). All 'with-bead' clean-up steps were performed using a generic SPRI substitute (Fisher *et al.*, 2011), 'speedbeads' (Faircloth *et al.*, 2015). During the ligation step of the library preparations we added either custom, single-indexing TruSeq-style barcode adapters (Faircloth *et al.*, 2012, 2015) or custom, dual-indexing TruSeq-style adapters (iTru; i5 and i7 primers) (Faircloth & Glenn, 2012). After ligation we PCR-amplified 50% of the resulting library volume (15 μL) with the following reaction mix: 25 μL HiFi HotStart polymerase (Kapa Biosystems, Wilmington, MA, U.S.A.), either 5 μL of Illumina TruSeq primer mix (5 μM) or 2.5 μL of each of Illumina TruSeq-style i5 and i7 primers, and 5 μL ddH_20, with the following thermal protocol: 98°C for 45 s, 10–13 cycles of 98°C for 15 s, 65°C for 30 s, 72°C for 60 s, and 72°C for 5 min.

Pooling. Following PCR, we purified the product using speedbeads and we eluted the purified DNA with 23 μL using a pH 8 elution buffer (EB). We checked the quality of the libraries by measuring DNA concentration with a Qubit 2.0 fluorometer broad range kit and running 2 μL of library on an agarose gel. Following the quality checks we pooled six to 10 libraries together at equimolar concentrations into enrichment pools and we adjusted pool concentrations to ~147 ng/μL using a vacuum centrifuge. Final concentrations of enrichment pools were 140–162 ng/μL.

Enrichment verification and size selection. We enriched each pool with 2749 custom-designed probes (MYcroarray, Ann Arbor, MI, U.S.A.), targeting 1510 UCE loci in Hymenoptera (Faircloth *et al.*, 2015). We followed the manufacturer's protocol for the MYcroarray MYBaits kit (Blumenstiel *et al.*, 2010), except that we used a 1:5 dilution of the standard MYBaits concentration, and we added 0.7 μL of 500 µM custom blocking oligos (designed for our custom sequence tags). Enrichment incubation was performed at 65°C for 24 h, after which we bound all pools to streptavidin beads (MyOne C1; Life Technologies) and purified enriched libraries according to a standard target enrichment protocol (Blumenstiel *et al.*, 2010). We PCR-enriched libraries using the 'with-bead' approach (Faircloth *et al.*, 2015). We combined 15 μL of the streptavidin bead-bound enriched library with 25 μL of HiFi HotStart Taq (Kapa Biosystems), 5 μL of Illumina TruSeq primer mix (5 μM each), and 5 μL of ddH_2O, and ran it at 98°C for 45 s, 18 cycles of 98°C for 15 s, 60°C for 30 s, 72°C for 60 s, and 72°C for 5 min. We purified the resulting reactions using 1.0X speedbeads and rehydrated the enriched pools in 22 μL EB. We quantified 2 μL of each enriched pool using a Qubit 2.0 fluorometer broad range kit (Life Technologies, Inc.).

Enrichment verification and size selection. We tested our enrichment by amplifying seven UCE loci with relative quantitative PCR (qPCR). For each of the seven loci we compared amplification profiles between enriched and unenriched pools (adjusted to the same concentration). We used a SYBR FAST qPCR kit (Kapa Biosystems) to perform qPCR on a ViiA 7 Real-Time PCR System (Life Technologies). Following enrichment verification, we quantified the DNA concentration of each pool using qPCR. We used the resulting concentrations to pool libraries at equimolar concentrations, creating a pool-of-pools. This final pool-of-pools was then size-selected to a fragment range of 250–800 bp using the BluePippin size-selection instrument (SageScience, Beverly, MA, U.S.A.). Size-selected library pools were sent to either the UCLA Neuroscience Genomics Core or Cornell's Institute of Biotechnology and sequenced as three partial lanes (each of the lanes included other samples) on an Illumina HiSeq 2500 (2 × 150 rapid run).

Phylogenetic analyses of single-gene data (ants and fungi)

We edited, aligned and concatenated the ant and the fungal sequences in GENEIOUS v9 (Kearse *et al.*, 2012) and compared

Table 1. Datasets.

Sequencing	Organism	Data matrix	Taxa number	UCE loci or genes	Description	Alignment length (bp)	Partitions	Distinct alignment patterns	UCE mean loci length
nextgen	Ant	50%	93	1062	50% complete matrix	873 481	29	259 630	873.481
nextgen	Ant	70%	93	1016	70% complete matrix	850 814	16	252 410	837.42
nextgen	Ant	80%	93	956	80% complete matrix	814 997	29	240 419	852.51
nextgen	Ant	90%	93	799	90% complete matrix	702 574	13, 799[a]	205 775	879.32
nextgen	Ant	95%	93	530	95% complete matrix	499 504	17	140 586	942.46
nextgen	Ant	ex1	92	816	One taxon excluded	716 032	31	206 593	877.49
nextgen	Ant	ex2	90	788	Three taxa excluded	695 579	33	197 562	882.71
nextgen	Ant	ex3	32	676	Only 32 taxa for PARAFIT included	625 196	25	60 826	814.56
nextgen	Ant	bs best all	93	100	100 UCE loci with highest bs values, all taxa	83 533	13	26 764	835.33
nextgen	Ant	bs best 15T	15	100	100 UCE loci with highest bs values, 15 taxa	88 157	11	8864	881.157
nextgen	Ant	random 1	15	100	100 random loci, for 15 taxa	90 771	12	8468	907.71
nextgen	Ant	random 2	15	100	100 random loci, for 15 taxa	94 740	13	8559	947.4
nextgen	Ant	random 3	15	100	100 random loci, for 15 taxa	90 760	13	8103	907.6
Sanger	Ant	COI	150	1	COI alignment	885	3	544	NA
Sanger	Fungal	ITS	81	1	ITS alignment	953	5	792	NA
Sanger	Fungal	ITS + LSU	93	2	ITS + LSU alignments	2329	6	1079	NA
Sanger	Fungal	LSU	54	1	LSU alignment	1412	3	351	NA

[a]The two numbers listed under 'number of partitions' for the 90% matrix indicate the results of PARTITIONFINDER analyses using the kmeans and greedy algorithms (the latter with UCE loci as data blocks), respectively.
Summary statistics for all datasets used in analyses. UCE, ultraconserved element; COI, cytochrome oxidase subunit I; ITS, internal transcribed spacer; LSU, large subunit.

our results with NCBI GenBank sequences using BLASTn (Altschul *et al.*, 1990; Johnson *et al.*, 2008) to check for possible contamination. We combined our newly generated sequences, ant COI and fungal ITS and LSU data, with previously published sequences downloaded from NCBI GenBank. The COI data included 125 specimens sequenced in this study, with the addition of 12 *Sericomyrmex* and 12 non-*Sericomyrmex* attine-ant GenBank sequences, the latter serving as outgroups. The fungal data included 32 *Sericomyrmex* fungal sequences generated in this study and 58 ITS and 25 LSU sequences downloaded from GenBank, consisting of both *Sericomyrmex* and non-*Sericomyrmex* fungal cultivars (Table S4). Numbers of taxa and other information for each dataset are summarized in Table 1. Alignments were generated with MAFFT v1.3.5 (Katoh & Standley, 2013) using default settings, as implemented in GENEIOUS v9 (Kearse *et al.*, 2012), and further refined manually. Three matrices were generated for the fungal data: one for ITS, one for LSU and one with both alignments combined (ITS + LSU). To select the best model of sequence evolution and data partitioning schemes for the COI dataset we used PARTITIONFINDER v1.1.1 (Lanfear *et al.*, 2012) and for all three fungal datasets we used PARTITIONFINDER v2.0 (Frandsen *et al.*, 2015). For COI we defined data blocks by gene and codon position and used the 'search = all' algorithm, whereas for the fungal datasets, which are from non-protein-coding regions, we used the 'search = kmeans' algorithm, which does not require predefined data blocks (Frandsen *et al.*, 2015). For each dataset we ran two PARTITIONFINDER analyses using 'AICc' model-selection criteria, one with 'models' set to 'raxml' and one with 'models' set to 'mrbayes'. The number of partitions and their models for each dataset and for each analysis can be found in supplemental material (File S2). We conducted simultaneous best-tree and rapid bootstrapping (1000 pseudoreplicates; −f a option) maximum likelihood (ML) analyses in RAXML v8.2.8 (Stamatakis, 2014). The resulting phylogenies were examined in FIGTREE v1.4.2 (Rambaut, 2009). We also conducted Bayesian analyses of all datasets using MRBAYES v3.2.6 (Huelsenbeck & Ronquist, 2001; Ronquist & Huelsenbeck, 2003). For each dataset we performed two runs (four chains per run) of 30–50 million generations. We confirmed that our runs reached stationarity and converged by making sure that the average standard deviation of split frequencies was below 0.01, by examining the estimated sample size (ESS) values for all parameters, and by comparing the two independent runs in TRACER v1.6 (Rambaut *et al.*, 2014). The number of generations and other computational details for each MRBAYES run are listed in supplemental material (File S2). The resulting consensus trees were examined in FIGTREE v1.4.2 (Rambaut, 2009). All phylogeny figures were prepared using FIGTREE v1.4.2 and Adobe ILLUSTRATOR CC.

Processing of UCE data

The raw sequence data were converted to FASTQ format and demultiplexed by the sequencing centres. We cleaned and trimmed raw reads using ILUMIPROCESSOR (Faircloth,

2013), which incorporates Trimmomatic (Bolger *et al.*, 2014). Following trimming, the data were processed through a series of scripts available in the PHYLUCE package v1.4 (Faircloth, 2015), with parallel processing on a 12-core Intel-processor Apple computer. We computed summary statistics for the trimmed FASTQ files (get_fastq_stats.py), assembled the trimmed reads with a script wrapper (assemblo_trinity.py) in the program TRINITY (version trinityrnaseq_r20140717) (Grabherr *et al.*, 2011), and calculated the coverage of all assembled contigs (get_trinity_coverage.py). After assembly we mapped Trinity contigs to UCE loci (match_contigs_to_probes.py, with settings: min_coverage = 50, min_identity = 80) and calculated coverage statistics for UCE contigs (get_trinity_coverage_for_uce_loci.py). We generated a list of UCE loci shared across all taxa (get_match_counts.py) and used this list to create a monolithic FASTA file containing all samples and sequence data (get_fastas_from_match_counts.py). We separated data by locus and aligned each locus using a wrapper script around MAFFT (Katoh & Standley, 2013). The resulting alignments were trimmed with Gblocks (Castresana, 2000) (get_gblocks_trimmed_alignment_from _untrimmed.py, settings: b1 = 0.5, b2 = 0.5, b3 = 12, b4 = 7).

After trimming, we created multiple datasets (summarized in Table 1) based on filtering UCE loci for differing levels of taxon occupancy (95, 90, 80, 70, and 50%) using the get_only_loci_with_min_taxa.py script. The 70% dataset, for example, contained only those UCE loci that included sequences for at least 70% of the taxa. We then added missing data designators ('?') for taxa missing from each alignment of a given locus (add_missing_data_designators.py) and generated matrix statistics for each dataset (get_align_summary_data.py).

UCE phylogenetic analyses

For data partitioning and model estimation of the UCE data, we used the kmeans algorithm in PARTITIONFINDER 2.0 (Frandsen *et al.*, 2015) which is designed to infer partitions and models directly, rather than through predefined data blocks (e.g. UCE loci), which is particularly useful for genome-scale data. Because of a concern, based on simulated data, that the kmeans search algorithm can produce odd results (P. Frandsen, personal communication), most probably associated with a large subset of invariant sites, we also ran a PARTITIONFINDER analysis in which UCE loci were defined as data blocks. We ran this analysis with the hcluster search algorithm on the 90% complete dataset. We conducted phylogenetic analyses of all UCE datasets under the ML criterion in RAXML v8.0.3 (Stamatakis, 2014) using a simultaneous best-tree search and rapid bootstrapping analysis (−f a option) with 100 bootstrap replicates. To investigate the effects of partitioning and number of bootstrap replicates on the results, we performed additional analyses on the 90% complete matrix in RAXML v.8.0.3 (also using the simultaneous best-tree search and rapid bootstrapping analysis): of an unpartitioned dataset (100 bootstrap replicates), of a dataset partitioned with UCE loci defined as data blocks (100 bootstrap replicates), and of a kmeans-partitioned dataset (500 bootstrap replicates). Datasets and some of the analysis details are summarized in Table 1.

In addition to concatenated analyses, we partitioned each UCE locus separately using PARTITIONFINDER 2.0 (using kmeans) and inferred partitioned gene trees with RAXML v.8.0.3 (simultaneous best-tree and rapid bootstrap search, 200 pseudoreplicates). We then carried out a species-tree analysis using the resulting gene trees as input into the program ASTRAL v4.7.6 (Mirarab *et al.*, 2014b), a species-tree method that uses a multi-species coalescent model to estimate the true species tree from unrooted gene trees. We ran the program with 100 multi-locus bootstrap replicates. ASTRAL has been shown to outperform similar methods in cases of rapid evolutionary radiations (Meiklejohn *et al.*, 2016).

To further explore the effects of possible gene-tree disagreement on our species-tree results, we performed statistical binning (Mirarab *et al.*, 2014). This method sorts UCE loci that agree topologically into bins, taking into account only well-supported bipartitions as determined by bootstrap proportions (Mirarab *et al.*, 2014a; Bayzid *et al.*, 2015), producing, for our data, 433 bins. We concatenated the loci from each bin into supergene alignments and used these alignments in gene-tree species-tree analyses using PARTITIONFINDER, RAXML and ASTRAL, as described earlier for the non-binned ASTRAL analysis.

We also used the individual gene trees as an input for the script gene_stats.R (Borowiec *et al.*, 2015) to calculated the average bootstrap support for each locus in R v3.1.2 (R Development Core Team, 2014) and created a dataset consisting of the 100 UCE loci with the highest bootstrap support (dataset bs_best_all) for all taxa.

To explore the effect of taxa with long branches, especially as some of them had lower coverages and read counts, we also created two reduced datasets, one (Ex1) from which *Sericomyrmex opacus* 1614 was excluded, and one (Ex2) from which *Sericomyrmex opacus* 1614, *Sericomyrmex mayri* 1555 and *Sericomyrmex amabilis* VE 1559 were excluded. We created these datasets by running the PHYLUCE pipeline from the get_match_counts.py step, with those taxa that we wished to exclude absent from the configuration taxon list. For these datasets we used only the 90% level of taxon filtering.

UCE divergence dating analyses

For divergence-dating analyses in BEAST v2.4.1 (Drummond *et al.*, 2012; Bouckaert *et al.*, 2014) we created four separate datasets, each with the same reduced numbers of taxa and UCE loci. In order to estimate species divergence dates using the Yule prior, in all datasets we reduced the number of taxa to a single representative per species, so that the dataset included 15 taxa (10 ingroup and five outgroup species). Using the script gene_stats.R (Borowiec *et al.*, 2015), we calculated the average bootstrap support for each locus in R v3.1.2 (R Development Core Team, 2014) and used the results to create a dataset consisting only of the 100 UCE loci with the highest bootstrap support (dataset bs_best_15T) from our 90% complete dataset. We used a reduced number of loci because BEAST analyses are

computationally demanding and larger datasets take intractably long periods of time to converge. To ensure that this subsampling of loci did not bias our results, we created three additional datasets consisting of 100 UCE loci (datasets random 1, random 2 and random 3), randomly subsampled using the function *sample* in R v 3.1.2 (R Development Core Team, 2014).

We calibrated two nodes (Table S5) using secondary calibrations taken from the most recent phylogeny of attine ants (M.G. Branstetter *et al*., unpublished data) because no fossils exist for *Sericomyrmex* or the outgroups included in this study, whereas M.G. Branstetter *et al*. (unpublished data) is calibrated with nine fossils and includes a much larger taxon sample from across the tribe Attini s.l. We used very broad normal distributions in our prior calibrations (Ho & Phillips, 2009) that encompassed more than the 95% highest posterior densities (HPDs) of M.G. Branstetter *et al*. (unpublished data) (Table S5) in order to account for the use of secondary calibrations, which are known to cause unrealistically narrow confidence intervals (Drummond & Bouckaert, 2015).

We conducted BEAST v2.4.1 analyses of all four datasets using unpartitioned data because of the much longer analysis times required for partitioned datasets and because of concerns that partitioned analyses do not produce consistent divergence dates (Dos Reis *et al.*, 2014; Zhu *et al.*, 2014; Ward & Fisher, 2016). However, we also performed one partitioned analysis, on the bs_best dataset only, to test whether partitioning had any effect on the results. We used the relaxed lognormal molecular clock, Yule tree prior and diffuse gamma distribution on the mean branch lengths (ucld.mean; alpha = 0.001, beta = 1000), with other settings at default, in all of our BEAST v2.4.1 analyses. For each dataset and for each analysis, we started two independent runs using the same xml file. In the first set of analyses we used the GTR model of evolution with base frequencies set to 'estimated' for 600 million generations and with log and tree sampling frequencies set at 5000 generations. The resulting log files had low (<100) ESS values for some of the statistics ('prior' and 'posterior') when examined in TRACER v1.6.0, even though the ESS for other parameters (e.g. likelihood of the tree) was high, and even though our runs converged on the same dates. Since overparameterization can cause low ESS values in BEAST v2.4.1 runs (Drummond & Bouckaert, 2015), and a complex model of evolution is one of the causes of this behaviour, we conducted a second set of analyses with a simpler model of evolution, HKY with base frequencies set to 'empirical'. We set up two independent runs for each of the four datasets consisting of 1 billion generations with log and tree sampling frequencies of 5000 generations. We also set up two independent runs for the partitioned bs_best dataset, with 500 million generations and with log and tree sampling frequencies of 1000 generations. For each dataset, two independent log files were combined in LOGCOMBINER v.2.4.1 (Bouckaert *et al.*, 2014). Examination of the combined log files in TRACER v1.6 (Rambaut *et al.*, 2014) indicated that ESS values were high for all parameters (>200) and that stationarity had been achieved by 100 million generations (burn-in of 10%). Because of computational limitations, we summarized the resulting tree files using a burn-in of 50% with TREE ANNOTATOR v2.4.1 (Bouckaert *et al.*, 2014) and examined them in FIGTREE v1.4.0. For each dataset we also combined two tree files from two independent runs, using LOG COMBINER, and summarized these combined trees with TREE ANNOTATOR v2.4.1.

To compare *Sericomyrmex* with other examples of recent radiations, we calculated the net diversification index (NDI), a time elapsed between the origin of a new lineage and the next branching of that lineage, assuming no extinction. NDI is defined as $t/(\ln Nt - \ln N_0)$, where t is the time to a single common ancestor in millions of years, Nt is a number of lineages, and N_0 the number of original species, which is one in monophyletic lineages (Coyne & Orr, 2004).

Analyses of coevolution

We tested for evidence of ant–fungus coevolution using PARAFIT, a permutation-based test for significant coevolution (Legendre *et al.*, 2002), as implemented in the R software package ape v.3.4 (Paradis *et al.*, 2004). For PARAFIT analyses we used the phylogeny resulting from the analysis of the combined fungal ITS + LSU datasets excluding outgroups and a phylogeny of the ant UCE loci, including only those ants that had associated fungi in the fungal phylogeny (dataset Ex3; Table 1). We exported the patristic distance matrices of both phylogenies from GENEIOUS v.9 and created a matrix of 32 associated ants and fungi. Six *Sericomyrmex*-associated fungi did not have an exact ant match because the fungus originated from a nest that was not represented in the UCE ant phylogeny. For four of those cases we associated the fungus with an ant of the same species from the same locality, and for the remaining two samples we associated the fungus with the same ant species but from a different locality. To determine whether the latter substitution biased our results, we created a second dataset with only 30 taxa, excluding the two associations with non-matching localities. Lists of taxa in both datasets can be found in Table S6. Analyses consisted of 999 permutations with the cailliez correction (Legendre & Legendre, 1998; Legendre *et al.*, 2002).

Results

UCE sequencing

The mean DNA sample concentration for our 93 taxa was 3.32 ng/μL (0.619–50 ng/μL) post-extraction, and 59.25 ng/μL (22.7–136 ng/μL) post-PCR libraries. The mean number of raw reads sequenced per sample was 1 447 502 (229 485–3 500 409) and the mean number of UCE loci captured per taxon was 994.23 (497–1054). Table 2 summarizes statistics for sequencing and pipeline processing (full statistics for each taxon are presented in Table S7). These statistics are similar to those in previous UCE studies using the same Hymenoptera probes (Blaimer *et al.*, 2015; Faircloth *et al.*, 2015). The total number of UCE loci in our concatenated datasets ranged from 100 to 1062. The most complete dataset, the 95% complete concatenated matrix, included 530 UCE loci.

Table 2. Ultraconserved element (UCE) sequencing statistics.

	DNA concentration of extract (ng/μL)	DNA concentration post-PCR library (ng/μL)	Raw read count	Contig number after trimming	Contigs coverage	Contig mean length	UCI loci coverage	Sequence coverage	Mean UCI loci length (bp)
Average	3.87	79	1 423 497.91	15 352.15	19.94	398.4	989.23	72.78	923.15
Minimum	0.619	17	299 485	4201	9.2	273.2	497	9.04	284.8
Maximum	50	136	3 500 409	71 228	31.7	768.4	1054	191.83	1218.4
Standard deviation	5.3	23.98	594 466.6	11 971.71	3.64	78.31	59.75	29.62	133.25

Mean, standard deviation, minimum and maximum sequencing and assembly statistics averaged for all 93 taxa in the ultraconserved element (UCE) dataset. For more detailed statistics per taxon, see supplemental material Table S6.

UCE phylogeny and species delimitation

Most datasets and analyses produced exactly the same topology and had bootstrap frequencies of 99–100 at all nodes, except one, for divergences at the species level and deeper (Fig. 2, Figure S1). The results indicate that the genus *Sericomyrmex* consists of two main groups, which we refer to as the *scrobifer* clade and the *amabilis* clade. This is the first such division to be discovered in *Sericomyrmex*, for which no species groups have been previously proposed. The *scrobifer* clade comprises three species: *S. scrobifer* and two new species, which we refer to as *S.* n. sp. 1 and *S.* n. sp. 2. All *scrobifer*-clade species occupy relatively long branches supported by bootstrap frequencies of 100 in all analyses. The larger *amabilis* clade includes five species: *S. opacus*, *S. parvulus*, *S. saussurei*, *S. amabilis*, *S. bondari* and *S. mayri*. It is highly likely, based on morphology (A. Ješovnik & T.R. Schultz, unpublished data), that the two *Sericomyrmex* species that are not represented in our UCE-based phylogeny, *S. lutzi* and *S.* n. sp. 3, also belong to the *amabilis* clade, with *S. lutzi* a close relative to *S. mayri*, and *S.* n. sp. 3 a close relative to *S. bondari*.

In an iterative process of species delimitation, each putative species-level clade in the UCE-based phylogeny was investigated with respect to geography and morphology. For geography, we studied distribution data to determine whether putative species-level clades could be interpreted as geographically distinct populations or as fully-fledged species. For morphology, we studied >1500 specimens, including type specimens, to determine whether putative species-level clades were characterized by discrete morphological character combinations. We have chosen not to recognize putative species-level clades as distinct species if: (i) they can alternatively be interpreted to be allopatric populations of a single widespread species; and (ii) they are morphologically indistinguishable from other populations of that widespread species. Based on these criteria, there are nine species-level clades present in our UCE-based phylogeny, including two new species. This number is lower than the currently recognized 19 species, even accounting for the two species that we were unable to include in the phylogeny, *S. lutzi* and another new species, *S.* n. sp. 3, which increase to 11 the total number of *Sericomyrmex* species.

A single specimen of *S. amabilis* from Venezuela occupies a long branch in the UCE phylogeny as the sister to the combined *saussurei* and *amabilis*, a position with maximum support in all analyses of all datasets (Fig. 2, Figures S1 and S3). This position, as well as intervening branch lengths, indicates that this specimen is molecularly distinct from the rest of the *amabilis* clade and could represent a new species. It is, however, morphologically indistinguishable from *S. amabilis* and is represented in our study by only three specimens, one destructively sampled for DNA extraction and two pinned. For these reasons, and because it is possible for one of two sister species to be paraphyletic with respect to the other (Coyne & Orr, 2004), we are for now treating *amabilis* VE as an allopatric population of *amabilis* that renders *amabilis* paraphyletic with respect to *saussurei*.

The only species-level clade supported by a low bootstrap frequency is *S. mayri* (68–100, depending on the analysis; Figure S1) and this is caused by the inclusion of a single rogue specimen, S. *mayri* 1555 TT from Trinidad and Tobago, which occupies differing positions in phylogenies produced by different datasets or analytical conditions. In phylogenies resulting from RAXML v8.0.3 analyses of the 50, 70, 80 and 90% complete concatenated matrices, *S. mayri* 1555 TT consistently occupies the position of the most basally diverging lineage in the *S. mayri* species-level clade (Fig. 2), although supported by low bootstrap frequencies (68–87). It likewise occupies this position in analyses of the 100_best_all dataset with a bootstrap frequency of 100. In ML analyses of the 95% matrix, however, *S. mayri* 1555 TT occupies the position of sister to *S. bondari*, while in both ASTRAL v4.7.6 species-tree analyses (of individual UCE loci and of binned UCE loci) it is recovered as the sister of the combined *S. bondari* and *S. mayri* clades, in both cases with a bootstrap frequency of 100 (Figure S1). The number of UCE loci sequenced for this taxon is average; however, some of the sequencing statistics are very low, including the number of raw reads and UCE locus length (Table S7), so its unstable position in some analyses could be an artefact of poor sequence quality and missing data. Considering all the evidence, we have chosen not to recognize *S. mayri* 1555 TT as a new species. Morphologically, it is indistinguishable from other members of S. *mayri* and, as summarized earlier, the UCE-based phylogenetic results are inconclusive. The phylogenetic position and species-level status of *S. mayri* in Trinidad certainly deserves further research; we were able to include only a single nest series in this study.

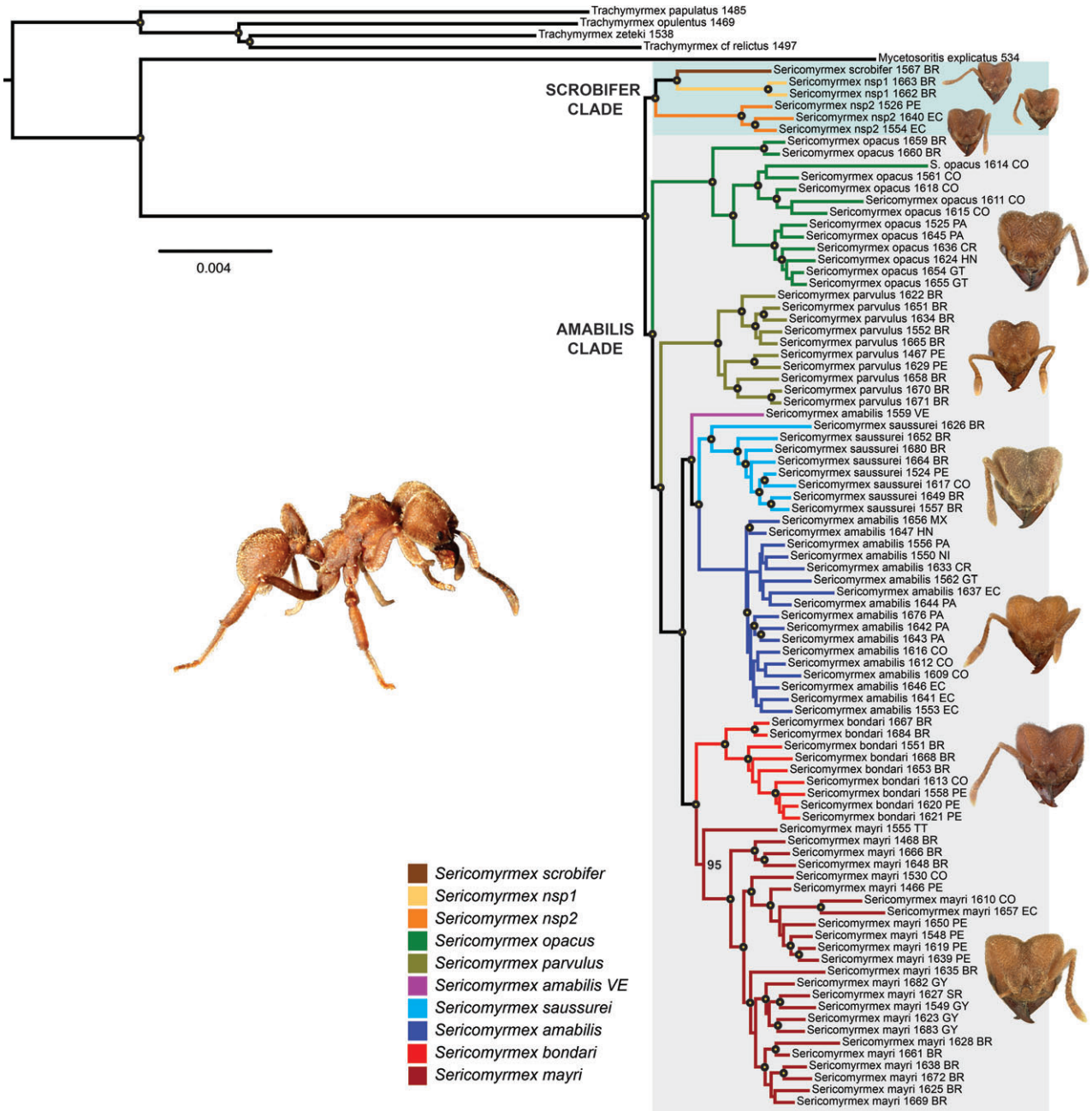

Fig. 2. Ultraconserved element (UCE) phylogeny. The maximum likelihood phylogeny of the 90% complete concatenated matrix containing 799 UCE loci (702 574 bp). Black circles at nodes indicate 98–100 bootstrap frequencies ($N = 500$). Taxon names include DNA extraction codes (numbers following species names) and country codes as follows: BR, Brazil; CO, Colombia; CR, Costa Rica; HN, Honduras; EC, Ecuador; GY, Guyana; GF, French Guiana; GT, Guatemala; MX, Mexico; NI, Nicaragua; PA, Panama; PE, Peru; SR, Surinam; TT, Trinidad and Tobago; VE, Venezuela. The two major *Sericomyrmex* clades are indicated with green (scrobifer clade) and grey (amabilis clade) coloured boxes.

In addition to *S. mayri* 1555 TT, we identified two other taxa as potentially problematic. *Sericomyrmex opacus* 1614 CO has very low sequence quality (Table S7) and occupies a relatively long branch. *S. amabilis* 1559 VE is likewise placed on a relatively long branch, outside of the *amabilis* + *saussurei* clade, even though it is morphologically identical to *S. amabilis*. We analysed two data subsets with these taxa excluded, Ex1 and Ex2 (Table 1). ML analyses of datasets with one (*S. mayri* 1555 TT) or all three of these taxa excluded resulted in phylogenies with the same topology as those obtained in most analyses (e.g.,

Table 3. Divergence date estimates.

Node	Average (all four datasets)	bs best 15T	bs best 15T partitioned	random 1	random 2	random 3
Sericomyrmex stem	**15.13 (8.56–21.85)**	15.63 (8.8–22.37)	15.63 (8.87–22.44)	15.41 (8.75–21.99)	14.73 (8.15–21.14)	15.29 (8.53–21.92)
Sericomyrmex crown	**4.32 (2.26–6.86)**	4.95 (2.47–7.54)	4.93 (2.49–7.54)	3.7 (1.92–5.5)	4.48 (2.29–6.9)	4.78 (2.36–7.49)
Sericomyrmex amabilis + *saussurei* crown	**2.33 (1.01–3.41)**	1.86 (0.87–2.93)	1.85 (0.88–2.94)	2.01 (0.99–3.08)	NA	2.64 (1.16–4.21)
Sericomyrmex amabilis VE crown	**2.77 (1.26–3.98)**	2.15 (1.05–3.35)	2.14 (1.03–3.33)	2.46 (1.27–3.72)	NA	3.1 (1.45–4.86)
mayri + *bondar*i crown	**2.37 (1.04–3.36)**	1.72 (0.81–2.73)	1.72 (0.81–2.73)	2.28 (1.16–3.47)	NA	2.45 (1.15–3.88)
mayri + *bondari* stem	**3.09 (1.36–4.46)**	2.42 (1.16–3.7)	2.42 (1.2–3.75)	2.77 (1.44–4.16)	NA	3.42 (1.46–5.5)
parvulus crown	**3.35 (1.67–4.98)**	3.21 (1.59–4.93)	3.2 (1.6–4.94)	3.35 (1.75–5.023)	NA	NA
opacus crown	**3.59 (2–5.96)**	4.29 (2.12–6.55)	4.26 (2.12–6.52)	3.59 (1.18–5.37)	NA	NA
S. nsp 2 crown	**4.03 (2.1–6.45)**	4.68 (3.27–7.2)	4.66 (2.35–7.18)	3.41 (1.77–5.1)	4.13 (2.06–6.35)	4.56 (2.2–7.11)
scrobifer + *nsp* 1 crown	**3.22 (1.63–5.26)**	3.92 (1.85–6.06)	3.91 (1.88–6.1)	2.84 (1.43–4.31)	3.42 (1.63–5.34)	3.42 (1.64–5.33)

Divergence dates estimated in BEAST analyses.

the topology in Fig. 1), with similarly high support values, indicating that the inclusion of these taxa does not have a negative effect on tree topology.

Divergence dating

All 10 BEAST v2.4.1 analyses, two for each of four different datasets and two for the partitioned dataset, recovered similar divergence dates (Table 3, Fig. 3). The dates recovered by the partitioned and unpartitioned analyses of the bs_best dataset were nearly identical (Table 3). The bs_best and random1 datasets recovered the same topology as the larger UCE datasets with the full number of taxa and the greater number of loci. The other two random datasets, random2 and random3, recovered topologies in which relationships within the *amabilis* clade were different, and were also different from each other (Figure S2). This is not surprising considering the small number of loci used in the BEAST analysis (100 loci versus 799 in 90% complete matrix). We used a reduced number of loci because BEAST analyses are computationally demanding and larger datasets take intractably long periods of time to converge. However, the nodes that we were most interested in dating, the stem and crown nodes of the genus *Sericomyrmex*, the stem and crown nodes of the two main clades, *amabilis* and *scrobifer*, and major internal nodes within the *scrobifer* clade, were dated similarly in BEAST analyses of all the datasets (Table 3). The mean estimates for *Sericomyrmex* stem and crown divergence times are 15.13 (95% HPD: 8.56–21.85) and 4.32 (95% HPD: 2.26–6.86) Ma, respectively (Fig. 4). These dates, especially the stem-date estimate, are younger than mean estimates in a recent transcriptome and genome data study, which were 22.6 and 4.9 Ma (Ješovnik *et al.*, 2016) for the same nodes. This discrepancy can be explained by taxon sampling; including the sister taxon *M. explicatus* in this study breaks the branch connecting *Trachymyrmex* and *Sericomyrmex*. The similarity between the dates in the current study and those in Branstetter *et al*., which are just slightly older, 16.18 and 4.71 Ma (M.G. Branstetter *et al*., unpublished data), are no doubt partly due to the overlapping UCE data in the two studies and to the use of dates from Branstetter *et al*. as secondary calibration priors in the current study. However, a large number of taxa, both attine ants and outgroups, included in Branstetter *et al*. were not included in this study, and, similarly, most of the *Sericomyrmex* species included in this study were not included in Branstetter *et al*., which included only five *Sericomyrmex* species. Within the *amabilis* clade, the most recent divergences occur between the sister species *amabilis* and *saussurei* (2.33 Ma) and *mayri* and *bondari* (2.37 Ma). Within the *scrobifer* clade, species divergences are somewhat older: *scrobifer* and *S.* n. sp. 1 share a most recent common ancestor at 3.4 Ma, and together they share a most recent common ancestor with *S.* n. sp. 2 at 4.19 Ma.

COI ant phylogeny

We generated COI sequence data for 125 *Sericomyrmex* specimens in order to: (i) inform taxon selection for the UCE dataset; (ii) provide additional information for a taxonomic revision and for understanding geographic distributions; and (iii) assess the usefulness of COI for species identification. Combined with the sequences downloaded from GenBank, the COI dataset consisted of 150 taxa and 885 bp of aligned sequence data (Table 1). Bayesian and ML analyses recovered similar phylogenies. Unsurprisingly, relationships between species were not congruent with those in the UCE phylogeny, and support values were very low across the entire COI tree. However, the two phylogenies generally agreed at the species level, i.e. in most cases terminal taxa grouped together into the same species-level clusters in both phylogenies (Figure S3). The exceptions are *S. mayri*

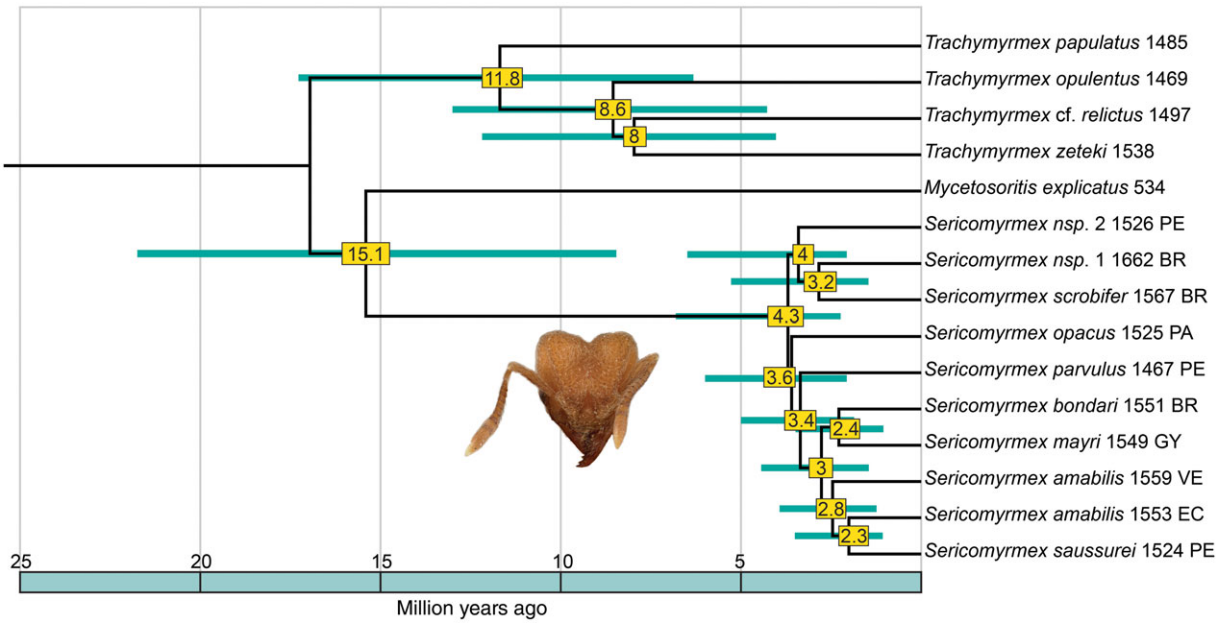

Fig. 3. Chronogram. The dated phylogeny resulting from a BEAST analysis of the ultraconserved element (UCE) data, with mean dates indicated in yellow boxes on nodes, calculated from all ten runs for all four datasets (Table 3). Green bars on nodes represent 95% highest posterior densities. [Colour figure can be viewed at wileyonlinelibrary.com].

and *S. bondari*, which were not recovered as monophyletic in the COI-based phylogeny but instead overlapped with each other with regard to some taxa, and *S. opacus*, which was recovered as paraphyletic.

We were particularly interested in the phylogenetic position of *Sericomyrmex* n. sp. 3 VE, a sample from Rio Negro, Venezuela, because of its distinct morphology and because we did not obtain UCEs for this specimen. We had only nine, dry-preserved, museum specimens greater than 30 years old, which, based on morphology, certainly represent a new species. Recent work has demonstrated that acceptable UCE sequences can be obtained from old, pinned, museum specimens (Blaimer *et al.*, 2016b), but unfortunately this information was published subsequent to the molecular phase of our study. In the COI phylogeny, *Sericomyrmex* n. sp. 3 is the sole occupant of a very long branch and, depending on the analysis, it groups with different clades, always with very low support (Figure S3). The uniqueness of the COI sequence corroborates our morphology-based conclusion that it represents a new species. Even though this sequence does not contain any stop codons, it is also possible that, due to degraded DNA, we sequenced a nuclear pseudogene of mitochondrial origin (*numt*) instead of the targeted fragment of COI (Kronauer *et al.*, 2007; Martins *et al.*, 2007; Cristiano *et al.*, 2014). In either case, we currently lack the molecular data for confidently estimating the phylogenetic position of *S.* n. sp. 3.

Fungal phylogeny and coevolution

We successfully sequenced one or both fragments of the ITS and LSU ribosomal nuclear gene regions for 32 fungi (28 from fungus garden samples and four from ant DNA extracts) associated with six different *Sericomyrmex* ant species and combined them with 58 ITS and 25 LSU sequences downloaded from GenBank (Table S4). Fungal phylogenies resulting from analyses of three different datasets (ITS, LSU, ITS + LSU) and two different analytical methods (ML and Bayesian) have similar topologies.

The phylogeny produced by the ITS + LSU and ITS-only dataset resulted in higher support values than those based on LSU-only datasets, a pattern observed in analyses of other fungal taxa (Schoch *et al.*, 2012), although LSU seems to perform better in analyses of fungi associated with lower-attine ants (Schultz *et al.*, 2015). The poorer performance of the LSU-only dataset is probably due to the low levels of divergence in LSU among higher-attine fungi.

The fungal phylogeny indicates that *Sericomyrmex* fungi are higher-attine fungi and that they belong to the same clade as all the *Trachymyrmex* fungi included in our analysis, in agreement with previous studies (Chapela *et al.*, 1994; Schultz & Brady, 2008) (Fig. 4). *Sericomyrmex* cultivars are not monophyletic, but instead are interspersed with fungi cultivated by *Trachymyrmex* species. The most derived *Sericomyrmex* cultivar species, which we call the *amabilis-mayri* fungus, is cultivated by the majority of *Sericomyrmex* species, including most *S. amabilis* from Central America, all of *S. mayri* from across its range, all *S. parvulus* and some *S. bondari*. This fungus is also grown by three *Trachymyrmex* species included in our phylogeny. A separate, small fungal clade, most closely related to the *amabilis-mayri* fungus, is cultivated by *S. amabilis* in Ecuador and a single *Sericomyrmex* from GenBank from an unknown locality (GU202430).

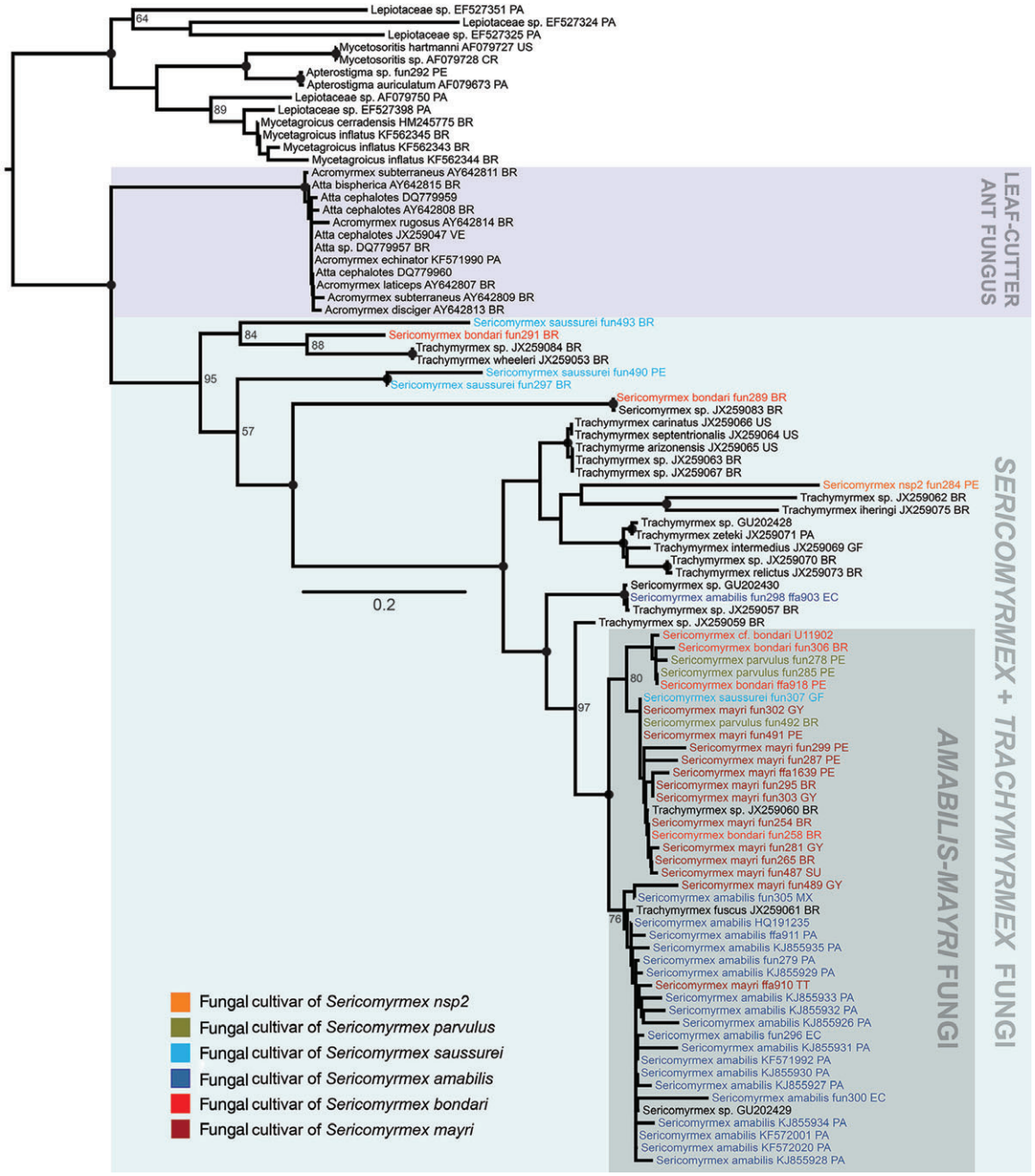

Fig. 4. Fungal phylogeny. The maximum-likelihood phylogeny of the ITS+LSU data set. Black circles at nodes indicate 98–100 bootstrap frequencies. Only values of >50% bootstrap frequency are indicated. Terminal taxa are named by their ant host species or genera except for free-living Lepiotaceae. Names of sequences generated for this study follow the color scheme of Fig. 2 and have a fungal extraction number of the form "fun123" following the taxon name, whereas sequences downloaded from GenBank are listed in black and have the GenBank accession number following the taxon name. All sequences for which the collection locality is known have a two-letter country code following the taxon name as follows: BR – Brazil, CR – Costa Rica, EC – Ecuador, GY – Guyana, GF – French Guiana, MX – Mexico, PA – Panama, PE – Peru, TT – Trinidad and Tobago, US – United States, VE – Venezuela.

The three most basally diverging, less inclusive lineages within the *Sericomyrmex-Trachymyrmex* fungal clade are fungi cultivated by *S. bondari* and *S. saussurei*, as well as a fungus cultivated by *Trachymyrmex opulentus* (= *wheeleri*), joined by long branches (Fig. 4) and grown at multiple localities across Brazil and Peru. Both *saussurei* and *bondari* also cultivate the more derived *amabilis-mayri* fungal species, sometimes at the same localities where they cultivate the aforementioned

basally diverging fungal lineages. A single fungal cultivar (*Sericomyrmex* n. sp. 2 fun284 PE) collected from a nest of *Sericomyrmex* n. sp. 2 in Peru occupies a long branch that assumes different positions in different analyses, albeit with low support values. Although this fungus is clearly a member of the higher-attine fungi, it does not cluster closely with any of the sequences produced for this study or with any sequences from GenBank. This is interesting because it is the only representative of the fungi associated with *scrobifer*-clade ant species, and it prompts the question of whether *scrobifer*-clade ant species may cultivate a clade of fungal species distinct from those cultivated by species in their larger and more diverse sister clade.

Evidence for global coevolution was found to be significant at the 0.05 level ($P = 0.029$) in a PARAFIT analysis of the full ant–fungal association dataset (32 ant-fungal associations) (Table S6). However, when the dataset was reduced to 30 ant–fungal associations, coevolution was not found to be significant at the level of 0.05 ($P = 0.235$). This result is in agreement with a previously observed pattern of diffuse coevolution in which higher-attine ants are strongly associated with higher-attine fungi (Schultz & Brady, 2008), but in which ant–fungus associations within these groups are labile, i.e. in which fungi are apparently horizontally transmitted across closely related (Green *et al.*, 2002; Mikheyev *et al.*, 2007, 2008) and distantly related ants (Schultz *et al.*, 2015).

Discussion

Evolution of the genus Sericomyrmex: a recent radiation with a high speciation rate

The evolution of the genus *Sericomyrmex* was, until recently, poorly studied. The genus is known for its problematic taxonomy, mostly because species descriptions are old and confusing, the available key (Wheeler, 1916) is outdated and incomplete, and, with regard to morphological variation, most species look alike. Our analyses of the UCE data resulted in a fully resolved and well-supported phylogeny, which we used to define two species-groups within the genus (*scrobifer* and *amabilis*), and which guided our efforts to delimit species. The criteria we use to define species of *Sericomyrmex* are: (i) they form well-supported lineages in the UCE phylogeny; and (ii) they are morphologically distinct from other putative species (A. Ješovnik & T.R. Schultz, unpublished data). The number of species-level lineages in *Sericomyrmex* (11) was found to be lower than previously described (19), even after accounting for the discovery of three new species. It is possible that we failed to detect morphologically indistinguishable, cryptic species, especially given our incomplete knowledge of the full morphological character and character-state space. One possible candidate is *S. amabilis* VE, representing the distinct sister lineage of the *amabilis* + *saussurei* clade. The two available specimens of *S. amabilis* VE are morphologically identical to the specimens assigned to *amabilis*. Because of the small number of specimens, the lack of distinguishing morphological character states, and its allopatric distribution, and because of the biological reality that a species may be paraphyletic with respect to its sister species, especially during the early stages of speciation (Coyne & Orr, 2004), we are for now recognizing *S. amabilis* VE as an allopatric population of *amabilis* that renders *amabilis* paraphyletic. The collection of additional specimens may improve our ability to determine whether or not this population is a separate species.

Two species that we recognize, *S. opacus* and *S. mayri*, contain particularly well-supported subspecific clades, which could be alternatively interpreted as distinct species or as genetically distinct populations. Because these clades are morphologically indistinguishable and because their distributions are correlated with geography, we do not recognize them as separate species. It is likely, however, that they represent cases of incipient species. There is little doubt that improved sampling, especially in regions where our sampling was poor (e.g. Venezuela, Bolivia, Ecuador, Amazonian Colombia, and Brazil) would result in the discovery of populations or species that would clarify *Sericomyrmex* species boundaries and relationships.

Our results indicate that the current distribution of *Sericomyrmex* is the product of a recent and rapid radiation. *Sericomyrmex* ants inhabit a wide variety of habitats from northern Mexico to southern Brazil, including wet and dry forests, riverbanks, cerrado, and urban and agricultural habitats. Even though the number of species (11) is lower than previously described, this is a large number of species and a high rate of speciation given the very short time since the origin of the genus. The crown-group age estimate for the entire genus *Sericomyrmex* is recovered as 4.3 Ma. For comparison, sister-species pairs in the *Cyphomyrmex wheeleri* group are estimated to have diverged 5.3–7.0 Ma (Mehdiabadi *et al.*, 2012) and the crown-age estimate for the species pair *Mycetophylax simplex* and *M. conformis* is 6.6 Ma (Cardoso *et al.*, 2014). So in the time in which comparable groups gave rise to two species, *Sericomyrmex* gave rise to 11 or 12. The crown-age estimate for the lower-attine species *Mycocepurus goeldi* and its social parasite *M. castrator* (2.04 Ma) (Rabeling *et al.*, 2014) is similar to those of within-*Sericomyrmex* species divergences (2.3–4 Ma). However, in the case of *Mycocepurus*, this recent divergence is attributed to its social-parasite biology because social parasites are known to have faster evolutionary rates than non-parasite ant species (Bromham & Leys, 2005; Jansen *et al.*, 2010; Rabeling *et al.*, 2014). The NDI of *Sericomyrmex* is 1.8 (0.9–2.8) million years, which is comparable to that of cichlid fishes in Lake Tanganyika (NDI = 1.6–2.2 Ma) (Turner *et al.*, 2001). Known examples of higher speciation rates (lower NDI) than that of *Sericomyrmex* are rapid radiations associated with island biogeography (radiation of the genus *Drosophila* in the Pacific ocean: NDI = 0.88 Ma; Kambysellis *et al.*, 1995) or with geographic isolation and selection pressures generated by a host symbiont, as in the remarkable ant-nest beetle genus *Paussus* on Madagascar (NDI = 0.36) (Moore & Robertson, 2014).

Perhaps more remarkable than the number of species, however, is that the rapid radiation of *Sericomyrmex* into diverse habitats across a large swathe of Central and South America was accompanied by remarkably little molecular and morphological change. As a group, *Sericomyrmex* ants are morphologically

distinct, instantly recognizable at the genus level, and quite different from any other ant, whether attine or non-attine. This indicates that a large amount of phenotypic evolution took place initially, possibly generating now-extinct species, on the very long branch separating the stem node from the crown node, i.e. the branch separating the common ancestor shared with *M. explicatus* from the most recent common ancestor of extant *Sericomyrmex* species (Fig. 3), but that, following the origin of the novel *Sericomyrmex* habitus, morphological evolution slowed down dramatically. One possible explanation for this rapid burst of phenotypic evolution at the genus origin is genome duplication, because known *Sericomyrmex* chromosome numbers and genome sizes are very high in comparison to those in other fungus-growing and non-fungus-growing ants (Murakami *et al.*, 1998; Tsutsui *et al.*, 2008; Cardoso *et al.*, 2014), and a number of cytogenetic studies suggest that chromosome evolution is correlated with genus and species diversification in ants (Lorite & Palomeque, 2010; Cardoso *et al.*, 2014).

Another distinguishing trait of *Sericomyrmex* ants is that they do not nourish and cultivate actinomycete bacteria (*Pseudonocardia*) on their integuments (Fernández-Marín *et al.*, 2009). *Pseudonocardia* bacterial films are associated with all other attine ants with the notable exception of those in the leaf-cutting genus *Atta*, also the product of a recent burst of phenotypic evolution. Attine-associated actinomycetes secrete narrow-spectrum antibiotics that are thought to be employed by the ants to protect their fungus gardens from infection (Currie *et al.*, 1999, 2003, 2006; Poulsen *et al.*, 2003), among other purposes (Mattoso *et al.*, 2012). The ancestors of both *Atta* and *Sericomyrmex* certainly had such bacterial films (e.g. they are present in *M. explicatus*), so it is possible that the rapid bursts of phenotypic evolution that accompanied the origins of both genera have been correlated in some way with the secondary loss of the integumental microbial symbionts present in all other attine ants. In response to garden infections, both genera compensate for the lack of *Pseudonocardia* by increased use of metapleural gland secretions, which are known to contain wide-spectrum antibiotics (Fernández-Marín *et al.*, 2006, 2009).

Diffuse coevolution with generalized higher-attine fungi

Sericomyrmex fungi belong to the clade of higher-attine fungi, distinct from lower-attine fungi by their polyploidy, their obligate rather than facultative symbiont status (Nygaard *et al.*, 2016), and their consistent production of gongylidia, swollen hyphal tips harvested by the ants for food [but see Masiulionis *et al.* (2014) for gongylidia-like structures in a lower-attine ant]. Our results indicate that *Sericomyrmex*-associated fungi are typical higher-attine ant fungi, a clade of multiple fungal species cultivated interchangeably between species of *Sericomyrmex* and *Trachymyrmex*. We find no evidence of tight (i.e. species-to-species) coevolution between *Sericomyrmex* ants and their fungi. Rather, our fungal phylogeny indicates the horizontal exchange of cultivars between *Sericomyrmex* and *Trachymyrmex* ant species (Fig. 4, Figure S4). However, associations between ants and their fungal cultivars do not appear to be entirely random. Most *Sericomyrmex* and some *Trachymyrmex* species and populations in our study grow a single fungal species, which we refer to as the *amabilis-mayri* fungus species. This species may have arisen relatively recently, but it has a large geographic distribution (Mexico to southern Peru and Brazil) and it is grown by all populations of *S. amabilis*, *S. mayri* and *S. parvulus* species included in our study. As with the leaf-cutting ant fungus species *Leucoagaricus gongylophorus*, which is also recently diverged and widely distributed (Aylward *et al.*, 2013), this pattern prompts the question of whether this particular fungus species is somehow better adapted for life with ants than other higher-attine fungus species, for example whether it is nutritionally superior, and, if so, why it has not replaced all the others. We know that nutritionally superior attine fungi promote, at least in the short term, the evolution of larger, ecologically dominant colonies (Mueller *et al.*, 2008, 2011; Nygaard *et al.*, 2016; Shik *et al.*, 2016) (with *Apterostigma megacephala* as a notable exception; Schultz *et al.*, 2015). In the long term, however, poorly understood ant–fungus incompatibilities can reduce the fitness of some ant–fungus species pairs (Seal & Mueller, 2014), possibly explaining why some fungi may replace resident fungi and spread to dominance across some species, but not others. Along these lines, it would be interesting to test whether the species of *Sericomyrmex* and *Trachymyrmex* that are most ecologically successful are consistently associated with the same, presumably more productive, fungal species.

UCE loci as a tool for species delimitation

Our results indicate that UCEs encompass variation sufficient for capturing recent species- and population-level divergences in *Sericomyrmex*. We conclude that UCEs represent an efficient choice for multi-level questions in insect phylogenomics, including species delimitation in recent and rapid radiations. A distinct advantage of UCEs as phylogenomic markers is the wide range of variation they encompass, ranging from the slowly evolving sites adjacent to the invariant core to increasingly variable sites in the flanking regions. This range of variability facilitates the incorporation of data across multiple phylogenetic studies carried out at multiple phylogenetic levels, obviating the need to resequence taxa for different markers.

The UCE-based phylogeny recovered nine *Sericomyrmex* species with strong support. In comparison, the COI phylogeny recovered six of those species, but failed to recover *S. bondari*, *S. mayri* and *S. opacus*. The obvious advantage of COI compared with phylogenomic markers is its affordability, but the disadvantage is that, judging by its success rate in *Sericomyrmex*, it is inaccurate a significant proportion of the time, probably due to incomplete lineage sorting, hybridization and other known problems (Brower, 2006; Rubinoff *et al.*, 2006). For large-scale projects with many specimens that are not concerned with correctly recovering supraspecific relationships, COI may be useful for delimiting species, but our results indicate that it should be used with caution and only in combination with other lines of evidence, such as morphology and geography. As a species identification (DNA barcoding) tool, however, our results indicate

that COI performs well, i.e. with the exception of *S.* n. sp. 3, COI-based species identifications referencing our archived COI sequence data (KY202274–KY202398) are expected to be accurate (Figure S3).

Supporting Information

Additional Supporting Information may be found in the online version of this article under the DOI reference: 10.1111/syen.12228

Figure S1. Additional UCE phylogenies. Phylogenies from all datasets: 50, 70, 80, 90–unpartitioned, 90%–UCE–partitioned, 95%, bs_best_all, ASTRAL analysis of individual UCE loci and of binned UCE loci.

Figure S2. BEAST phylogenies for each dataset (bs_best, bs_best partitioned, random 1, 2, and 3).

Figure S3. COI ant phylogeny. Maximum likelihood tree from RAXML and MRBAYES analyses.

Figure S4. Associated ant and fungal phylogenies. The maximum likelihood phylogenies of the reduced ant UCE dataset, including only ants for which associated fungi were available, and of the fungal ITS + LSU dataset, including only *Sericomyrmex* fungi.

Table S1. List of ant specimens. Collecting information, extraction codes, voucher specimen numbers, and GenBank and SRA accession numbers.

Table S2. List of fungal specimens. Collecting information, extraction codes, voucher specimen numbers, and GenBank accession numbers.

Table S3. Primers and PCR protocols. Primers and protocols used for Sanger sequencing of ant COI and fungal ITS and LSU.

Table S4. List of sequences downloaded from GenBank. Ant COI and fungal ITS and LSU, with locality information when available.

Table S5. Calibrations for ant divergence dating in BEAST.

Table S6. Ant–fungus association table with global and individual PARAFIT results, per taxon, for both PARAFIT runs. Grey-highlighted cells indicate a significant result at $P < 0.05$.

Table S7. UCE sequencing statistics for all taxa.

File S1. Speedbeads cleaning protocol.

File S2. Single-gene analyses details.

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Data availability

Quality-trimmed sequence reads generated in this study are available from the NCBI Sequence Read Archive under the Bioproject ID PRJNA354064, sample numbers SAMN0604441–SAMN0604533 (Table S1). Single gene sequences are available on GenBank under accession numbers KY173361–KY173382 (ITS), KY176772–KY176799 (LSU), KY202274–KY202398 (COI), also listed in Table S1.

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