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High diversity and multiple invasions to North America by fungi grown by the northern-most *Trachymyrmex* and *Mycetomoellerius* ant species

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ABSTRACT

Lower diversity at range margins of expanding populations is thought to reduce host-symbiont specificity of obligate symbioses. Selection for relaxed symbiont recognition systems is thought to occur when most, if not all, symbionts available to a host are genetically similar. This study evaluated whether the genetic diversity of symbiont populations along a range margin (North America) were lower than those of Neotropical populations in fungus gardening ants (subtribe Attina). Using phylogenetic, population-genetic and community-ecology approaches, we tested the hypotheses that North American fungal populations are genetically less diverse than Neotropical populations and whether they exhibit evidence of recent population expansion. Results indicated that fungal diversity is somewhat greater in the Neotropics than North America; however, North American populations are very diverse because all lineages found in the Neotropics are also represented in North America. Moreover, we found evidence of recent population expansion in both the Neotropics and North America.

1. Introduction

When obligate symbioses expand into a new unoccupied territory, both host and symbiont must successfully disperse into the new region. Otherwise, in the absence of a suitable partner, the symbiosis will fail. Population expansion of obligate symbiosis is further hampered by genetic bottlenecks and reduced genetic variability associated with expanding population fronts (Sexton et al., 2009; Hill et al., 2011; Smith et al., 2019). It is hypothesized that lower diversity along expanding range margins could result in relaxed host-symbiont associations (specificity) and possible limits to adaptation to local or changing environments (Douglas, 2010). For example, reduced symbiont diversity may relax the need for recognition systems because hosts will likely encounter genetically similar individuals, if the environment lacks diversity of hosts and symbionts due to patchiness or isolation in marginal environments (Funk and Bernays, 2001; Douglas, 2010).

Fungus-gardening or 'attine' ants (Formicidae: Myrmicinae: Attini:Attina) are obligately dependent on specific fungi that serve as the ants' primary digestive organ. The symbiosis is thought to have evolved 55–65 mya in what is now Amazonia and has since expanded well into the northern and southern temperate zones (Nygaard et al., 2016; Branstetter et al., 2017; Mueller et al., 2017; Smith et al., 2019). The fungus-gardening ants (subtribe Attina) have been considered a textbook example of diffuse coevolution because generally broad clades of ants grow certain clades of fungi, even though there may be host switching within clades or rare switches among clades (Mikheyev et al., 2006; Schultz and Brady, 2008; Schultz et al., 2015; Branstetter et al., 2017; Mueller et al., 2018). The exact mechanisms that determine or prevent host switching or how these mechanisms may change during range expansions are largely unknown.

The Fungal Control Model (FCM) posits that symbiont fidelity is enforced by the ants that recognize and prefer compounds produced by the fungus, so that the ants will reject non-native fungi

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(competing strains) from the nest (Bot et al., 2001; Poulsen and Boomsma, 2005; Poulsen et al., 2009; Seal et al., 2012). Under this model, ants can be forced to grow non-native fungi by removing their native garden and providing them new fungus over the course of several days, and the ants will eventually accept the novel fungus as their own as the hypothesized incompatibility compounds are purged from the system (Poulsen and Boomsma, 2005). However, research on North American species and Panamanian species over the past 1-2 decades has not shown unequivocal support for the FCM. Cross-fostering experiments, where North American fungus-gardening ants in the genus *Trachymyrmex* were provided with distantly related fungi from leaf-cutting ants nests (*Atta* or *Acromyrmex*), rarely resulted in rejection behavior, and ants frequently could be forced to grow novel fungi (Seal and Tschinkel, 2007; Seal et al., 2012, 2014; Seal and Mueller, 2014). These results were very surprising because the genetic distances between fungi typically grown by *Trachymyrmex* ants (so-called 'Clade-B' fungi) and leaf-cutting fungi ('Clade-A' fungi) (Mueller et al., 2018) are much higher than the genetic distances among the fungal symbionts examined in the studies that developed the FCM (Poulsen and Boomsma, 2005; Kooij et al., 2015b). One possibility is that the FCM may not apply to North American populations because more extreme temperate conditions and the lower diversity of fungal symbionts at range margins may select against incompatibility factors, leading to greater acceptance of non-native fungi and thus reduced specificity of ant-fungus associations (Kooij et al., 2015b). Consequently, lower diversity of fungal symbionts cultivated by North American attine ant species may select for a reduction or relaxation of well-developed symbiont recognition systems, which theoretically would not be needed if all symbionts are similar to one another and can, therefore, be exchanged readily between ant species near the range margins (Douglas, 2010).

While there have been several phylogenetic studies that have included fungi from North America and the tropics, there have been no comprehensive sequence analyses of fungi grown by North American and tropical *Trachymyrmex* ants (Mikheyev et al., 2008; Semenova et al., 2011; De Fine Licht and Boomsma, 2014; Ješovnik et al., 2017; Mueller et al., 2018). Thus, it is not known if ant fungi invaded North America only once or multiple times, and whether there might be distinct North American fungal lineages as found among Clade-A fungi (Mueller et al., 2011, 2017). The genus *Trachymyrmex* has been recently reorganized into three genera (Solomon et al., 2019). Accordingly, most North American *Trachymyrmex* ant species belong to *Trachymyrmex* sensu stricto that represent the sister lineage to the monophyletic leaf-cutting ants (*Atta* and *Acromyrmex*) (Schultz and Brady, 2008; Solomon et al., 2019). Two other North American species formerly in *Trachymyrmex* sensu lato are now classified (along with many tropical species) into the new genus *Mycetomoellerius*. *Mycetomoellerius jamaicensis* is a Caribbean species that barely reaches South Florida and *Mycetomoellerius turrifex* is a very common species in much of Texas and western Louisiana and northeastern Mexico (Rabeling et al., 2007; Sanchez-Peña, 2010; Seal et al., 2015; Senula, Seal and Kellner, unpublished data). It might therefore be possible that, because most North American ants belong to *Trachymyrmex* sensu stricto, they may grow fungi that are distantly related to the fungi of *Mycetomoellerius* ants in North America. *Trachymyrmex septentrionalis* is known to grow up to four phylotypes of fungi across its range (Mikheyev et al., 2008), which suggests that the North American attine fungal community could be quite diverse. The goals of this study were to: (1) compare the diversity of fungi grown by *Trachymyrmex* and *Mycetomoellerius* ants in North America with fungi grown by Clade-B growing ants (*Mycetomoellerius*, *Paratrachymyrmex* and *Sericomyrmex*) found in the tropics proper (Central and South America); and (2) examine whether the diversity patterns in North America show signatures of recent population expansion (e.g., reduced richness towards the range margin).

2. Materials and methods

2.1. Taxon sampling

Trachymyrmex septentrionalis, *Trachymyrmex pomonae, Trachymyrmex arizonensis* and *M. turrifex* colonies were collected from sites across the southern United States, with intensive sampling around central and eastern Texas (Fig. S1 A and B). Queens (if found), workers, and fungi were collected from each colony by careful excavation (Seal and Tschinkel, 2006). Colonies were then brought to the laboratory and housed in plaster-lined plastic boxes, and were watered and fed weekly before genotyping. Additional sequences of fungi cultivated by *Trachymyrmex, Mycetomoellerius, Paratrachymyrmex* and *Sericomyrmex* species in other regions of the United States and in the Neotropics were acquired from GenBank (Table S1). Fungal sequences of *Leucoagaricus* and *Leucocoprinus* fungi not associated with an ant host were not included in our analysis. We also included Clade-B fungi grown by leaf-cutting ants (*Atta* and *Acromyrmex*) in South America (Mueller et al., 2018) in the population-genetic analyses. We refer to sequences collected in the United States as 'North America', since the only sequences at GenBank collected in Mexico were in southern (tropical) Mexico.

Our dataset included 358 sequences, which contained 106 sequences (105 from GenBank) from fungi isolated from Neotropical fungus-gardening ant species (De Fine Licht and Boomsma, 2014; Kooij et al., 2015a; Ješovnik et al., 2017) and 252 sequences from North American fungal cultivars that were either obtained directly from field collections as part of this study, from unpublished sequences obtained by the senior author (Seal and Mueller, 2014; Seal et al., 2014) (N = 224), or from published sequences on GenBank (N = 28) (Mikheyev et al., 2008). Most new sequences reported here were collected from the western Gulf Coastal Plain of North America (Noss et al., 2015). This region corresponds to the zone of overlap between *M. turrifex* and *T. septentrionalis* (Rabeling et al., 2007; Seal et al., 2015). We also included sequences from fungal cultivars collected in southeastern Arizona from *T. arizonensis* and *T. pomonae* colonies (Table S1; Fig. S1).

2.2. DNA sequencing

DNA extraction and sequencing followed established protocols (Seal and Tschinkel, 2007; Sen et al., 2010; Seal et al., 2012). Fungal samples were collected using flame-sterilized forceps from the laboratory colonies for genotyping. Gongylidia were separated from fungal garden samples under a dissecting microscope and placed in 200 μl of a 20% Chelex (Sigma-Aldrich) solution and nuclease-free water. Samples were incubated for 1.5 h at 60 °C followed by 15 min at 99 °C. The supernatant was then diluted tenfold.

Primers ITS4 (5′−TCCTCCGCTTATTGATATGC−3′) and ITS5 (5′−GGAAGTAAAAGTCGTAACAAGG− 3′) were used to amplify ~600 bp of the internal transcribed spacer (ITS) regions and the intervening 5.8 S region, which are located between the highly conserved 18 S and 28 S rRNA genes (White et al., 1990). ITS has been used to differentiate major clades of fungus-gardening ant fungi (Mikheyev et al., 2008; Kellner et al., 2013; De Fine Licht and Boomsma, 2014; Ješovnik et al., 2017; Mueller et al., 2018). Although we sequenced only one gene, ITS produces tree topologies that are identical to those obtained from elongation factor 1 alpha (EF1α) or large ribosomal subunit (LRS) (Mikheyev et al., 2008; Ješovnik et al., 2017). Most studies that deposited fungal sequences in GenBank either analyzed ITS (de Fine Licht et al., 2014; Ješovnik et al., 2017) or ITS

and EF1α (Mikheyev et al., 2008; Mueller et al., 2018). Consequently, we focused our efforts on ITS sequences because of their greater availability in GenBank and in personal collections from prior studies (Seal et al., 2012; Seal and Mueller, 2014).

Polymerase chain reactions (PCRs) contained 1 μl of 10× PCR buffer (1.0×), 0.8 μl of deoxyribonucleotide triphosphates (1 mM each; 0.08 mM of total dNTPs as a proportion of the total master mix), 0.8 μl of 25 mM magnesium chloride (2 mM), 0.4 μl of 20 μM bovine serum albumin (0.8 μM), 0.6 μl of each 2 μM forward (ITS5) and reverse (ITS4) primer (0.12 μM each), 0.1 μl of *Taq* polymerase (0.05U), and 2 μl of fungal DNA template. Nuclease-free water was used to make up the remaining volume of the 10 μl reaction. The following thermocycling profile was used: 94 °C for 4 min; 35 cycles of 94 °C for 30 s, 51.5 °C for 45 s, and 72 °C for 1 min; 72 °C for 10 min. PCR products were visualized on 1% agarose gels with SYBR Safe gel stain. Products were then purified and sequenced at the University of Texas at Austin's DNA Sequencing Facility on an Applied Biosystems 3730 DNA Analyzer. Chromatograms were visually checked and resolved in Geneious R9 9.1.5 (Kearse et al., 2012), and sequences were aligned using MEGA 6.06 (Tamura et al., 2013), which was aided by an unpublished alignment provided by Ted Schultz (Smithsonian National Museum of Natural History). Sequences were deposited in GenBank under the accession numbers **MK142314-MK142538** (Table S1).

2.3. Phylogenetic analyses

Phylogenetic trees were reconstructed using both maximum likelihood (ML) and Bayesian inference (BI). We analyzed our sequences using ML as implemented in RAxML 8.2.10 (Stamatakis, 2014) and run on the CIPRES Science Gateway 3.3 (Miller et al., 2010) using default settings. The default GTR + G substitution model was applied, and node support values were obtained from 1000 non-parametric bootstrap (BS) replicates.

We also analyzed our sequences using BI in MrBayes 3.2 (Ronquist et al., 2012). For this analysis, we applied the best-ranked model of nucleotide evolution *a priori* selected in jModelTest (Posada, 2008) following the Bayesian information criterion; the model that best fitted the data was HKY. Two Markov chain Monte Carlo (MCMC) analyses with four chains each were run for 10, 000, 000 generations, sampling every 1000 generations. Bayesian posterior probabilities (PP) were calculated after confirming that the average standard deviation of split frequencies was lower than 0.01, and the first 25% of trees were discarded as burn-in. A leaf-cutter ant fungus (*Leucocoprinus gongylophorus*) from an *Atta texana* garden collected in western Louisiana was used as the outgroup.

Phylogenetic trees were visualized in FigTree 1.4.2 (Rambaut, 2009). Topologies from both the ML and BI were compared and combined into one phylogeny for visualization. The ML topology indicated two clades (Clades B2 and B3, see Population genetic analyses below for terminology), as sister taxa but with low support (BS = 55), whereas the BI topology suggested monophyly between these clades and two others (Clades B2−B5) that in turn formed a sister group to several early diverging lineages (Phylogroup 1) and had higher support (PP = 1.00). Consequently, the BI topology was followed (Fig. 1). We collapsed nodes if there was a shallow polytomy with a subtending branch supported with very high support values (i.e., PP > 0.9 or BS > 90), if sister taxa were from the same host and general location, or if there was ambiguous placement of Neotropical sequences (i.e., those from GenBank). The combined phylogeny was imported into Adobe Illustrator CS6 16.0.0 for annotation.

2.4. Population genetic analyses

While none of the Clade-B fungi have been formally described, unlike Clade-A (*L. gongylophorus* (Mueller et al., 2018)), we treated each of the major Clade-B linages as distinct species in our analysis. Clade-B fungal lineages are not thought to exhibit recombination among clades (Mikheyev et al., 2008). Due to the lack of terminology, we named each clade numerically (Clade B2, B3, etc.). Our naming scheme differed from that of Mikheyev et al. (2008) who termed the major fungal lineages as Clades A-D; to avoid confusion with the new names in Mueller et al. (2018) (Clades A and B), we referred to all clades and groupings as a subset of Clade B. Accordingly, Clade A of Mikheyev et al. (2008) corresponds to our Clade B4, Clade B is Clade B5, Clade C is Clade B2 and Clade D is Clade B3, respectively. The single exception was a group of early-diverging lineages whose relationships were inadequately resolved, hence the ambiguous term 'Phylogroup' (Fig. 1). We used intraspecific approaches to examine the population genetics of these fungal lineages. Specifically, we (1) addressed the population structure of the main fungal clades identified with the phylogenetic analysis and (2) examined the within-ant-host diversity of fungal populations.

We used Arlequin 3.5 to calculate the numbers of sequences, the number of unique sequences, and tests of population expansion for each clade/phylogroup of Clade-B fungi (Excoffier and Lischer, 2010). Specific tests employed were Tajima's *D* (Tajima, 1989) and Fu's *Fs* (Fu, 1997). The null hypotheses of Tajima's *D* and Fu's *Fs* are that the genetic diversity in populations has evolved neutrally, with an alpha level of 0.05 (*D*) or 0.02 (*Fs*) (Excoffier and Lischer, 2010). If populations do differ from neutrality, negative *D* or *Fs* values suggest an excess of low frequency polymorphisms/rare alleles, which may indicate population bottlenecks or recent population expansion, while significant positive *D* or *Fs* values suggest population contractions.

2.5. Descriptions of regional symbiont diversity

To describe the diversity of fungal symbionts found in North America and the Neotropics, we used two community ecology approaches. First, we conducted individual-based rarefaction analyses in EstimateS 9.1.0 (Colwell, 2013) to explore sampling effort and provide a more accurate comparison of the haplotype diversity in each of the two major regions for each fungal clade and, separately, for each host species. That is, we treated fungal clade or host species as individual "species" and haplotypes as "samples". We calculated the non-parametric, bias-corrected Chao1 estimator for each of these groupings using 100 randomizations and sampling without replacement. For some groupings, the Chao1 estimator could not be calculated because there was only one individual per haplotype. Second, to compare the weighted diversities of fungal haplotypes between regions, we calculated both the Simpson's diversity index (D (Simpson, 1949)) and the Shannon-Wiener function (H (Spellerberg and Fedor, 2003)) using the *diversity* function in the R 3.4.4 package *vegan* (Oksanen et al., 2018). We report 1-D, so values that are closer to 1 indicate higher diversity, while those closer to 0 indicate lower diversity. Simpson's index is more sensitive to dominance than Shannon-Wiener, thus lower 1-D values could indicate the presence of dominant individuals in communities. Diversity indices were only calculated in a large-scale comparison between North America and the Neotropics due to small sample sizes within fungal clades or within host species.

3. Results

3.1. Taxon sampling

Our dataset consisted of a total of 358 fungal sequences, including a Clade-A fungus from *A. texana* as an outgroup

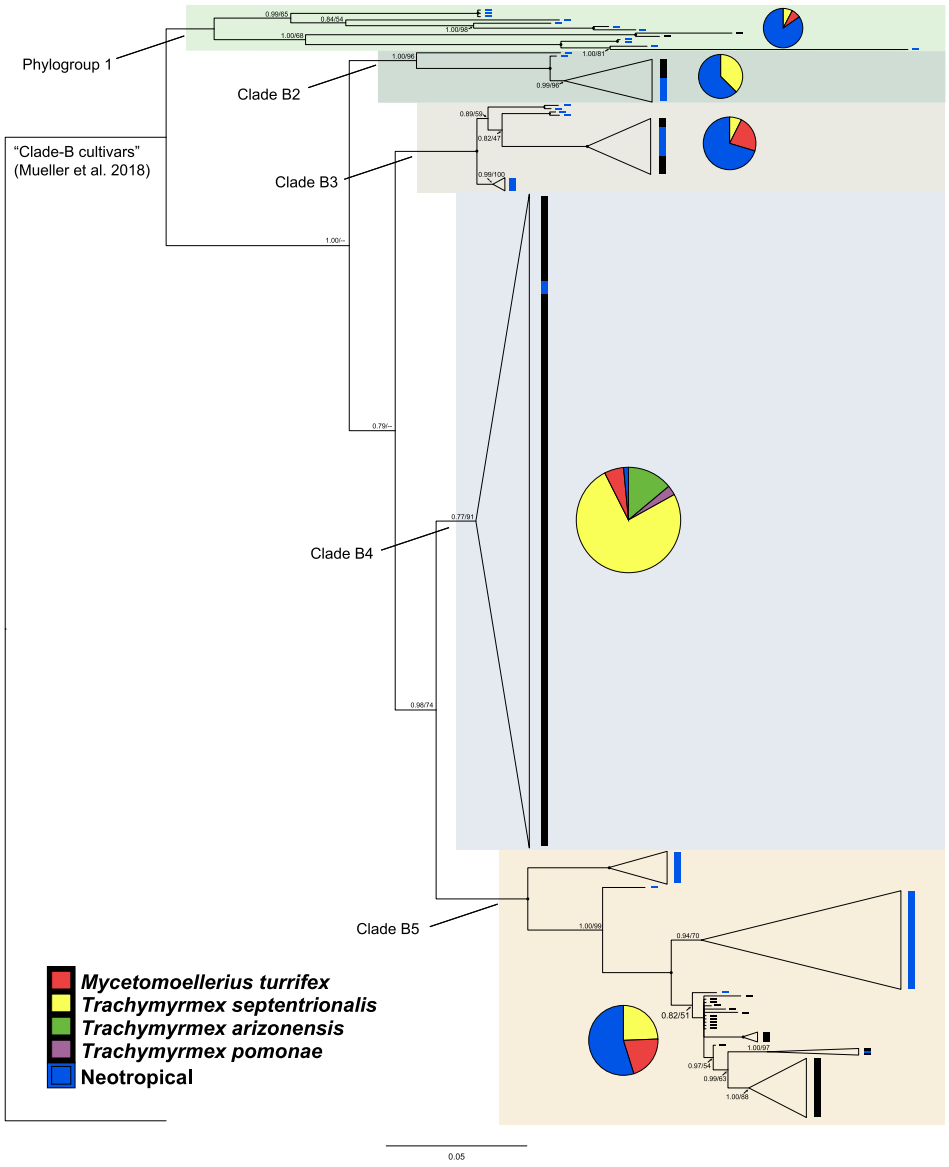

Fig. 1. Combined Bayesian inference and maximum likelihood molecular phylogeny of internal transcribed spacer "Clade-B" (Mueller et al., 2018) fungi cultivated by North American and Neotropical fungus-gardening ants. Numbers at the nodes refer to Bayesian posterior probability (PP)/maximum likelihood bootstrap support (BS), and "- -" indicates nodes in which topologies differed. Black circles indicate 1.00 PP and 100% BS. Nodes were collapsed if there was a shallow polytomy with very high support (i.e., PP > 0.90 or BS > 90), if sister taxa were from the same host and general location, and/or if there was ambiguous placement of Neotropical sequences (i.e., those from GenBank). Bars next to terminal tips indicate collection location (blue = Neotropics; black = North America). Colored pie charts represent the host species composition of each clade (Neotropical hosts are collectively "Neotropical") and are sized relative to the number of sequences in each major clade.

(Table S1). Collections that resulted from this study included 224 fungal symbionts from North American ant hosts and one fungal cultivar collected from a Neotropical species (*Mycetomoellerius zeteki*). To these sequences we added, from GenBank, 28 sequences from North American hosts and 105 sequences from Neotropical hosts (Mikheyev et al., 2008; Semenova et al., 2011; De Fine Licht and Boomsma, 2014; Ješovnik et al., 2017; Mueller et al., 2018). We included all available sequences from tropical ant hosts, except a single sequence collected from an undescribed species from Peru (*Sericomyrmex* n. sp. 2 fun284 PE; KY173370), which we excluded because of its ambiguous phylogenetic affinity and its inclusion reduced the support for many other branches in our analyses. Ješovnik et al. (2017) also observed that this specific sequence formed a long branch and did not group with any other sequences.

3.2. Phylogenetic analyses

The purpose of the phylogenetic analysis was to determine the placement of North American fungi in the context of all known Clade-B fungi cultivated by attine ants. Fungal taxa from North America were found throughout the phylogeny in all major radiations (Fig. 1, Fig. S2), and each of the major cultivar radiations had members from Neotropical and North American localities. Some of the major radiations of Clade-B fungi appeared more common in the tropics (e.g., Phylogroup 1, Clades B2 and B3), some were evenly split between tropical and northern temperate hosts (Clade B5), and some were predominately but not exclusively North American (Clade B4). While most North American fungi belonged to Clade-B4 (56%, N = 198 of 357 total; Table 1), two fungi from Brazil (grown by undescribed *Trachymyrmex sensu lato* species) and a third fungus

from Argentina (grown by an *Acromyrmex striatus* colony) were placed within the well-supported group Clade-B4. Samples from the Phylogroup were notably absent in the analysis by Mikheyev et al. (2008).

Most of the clades had high support values (Clades B2–B5; Fig. 1, Fig. S2). The single exception occurred among several early-diverging lineages that had long branch lengths; for example, lineages that contained *Mycetomoellerius opulentus* (a synonym of *Trachymyrmex wheeleri;* Mueller et al., 2017) and *Sericomyrmex bondari*, among others (Fig. 1, Fig. S2) (Solomon et al., 2019). There were two samples collected from Texas that clustered with these lineages. Bayesian analyses suggested monophyly but with low support, whereas maximum-likelihood analyses indicated several lineages that formed sister relationships to the remaining clades similar to the topology reported from an analysis of ITS and LRS genes (Ješovnik et al., 2017). However, an earlier analysis of ITS and EF1α produced a topology that suggested an early diverging monophyletic group of these lineages, though this topology also lacks strong support (Mueller et al., 2018). Because of the lack of agreement and/or the lack of support among studies and analyses, we refer to these lineages with the less stringent term of 'Phylogroup'.

The overall radiation patterns suggests that Phylogroup 1, Clades B2, B3 and B5 diverged in the tropics with some lineages radiating into North America, because North American fungi radiated from within larger tropical clades, though support is low for clear distinction between North American and Neotropical populations (Fig. 1, Fig. S2). For example, while North American Clade B5 fungi mainly comprises a monophyletic clade that branches off from a mainly Neotropical stem lineage, a single Neotropical collection (a fungus from *Sericomyrmex amabilis* from Panama) arises from within the large clade of otherwise North American taxa. Similarly, North American Clade B2 fungi are nested within a larger clade that contains Neotropical members.

There also appears to be very little support for ant-fungus clade-to-clade correspondences at the level of the well-supported fungal clades, because there are several cases of fungi from the same ant host species that are placed into multiple subclades throughout the fungal phylogeny. North American and tropical ant species exhibited considerable among-clade diversity of their farmed fungi. For example, *T. septentrionalis, M. turrifex, Paratrachymyrmex cornetzi*, and *M. zeteki* all grow fungi belonging to 4–5 clades (Table 2). Exceptions to this include some *Sericomyrmex* ant species that appear to cultivate fungi restricted largely to one fungal clade such as Clade B5 (*S. amabilis* and *Sericomyrmex mayri*), whereas other fungi from *Sericomymrex* are found associated with Phylogroup 1 fungi (Fig. 1, Fig. S2; Ješovnik et al., 2017).

3.3. Population genetic analyses

Examination of within-clade 'population' ITS diversity further suggested greater fungal diversity in the tropics compared to North America, but also suggested that much of this pattern was driven by unequal representation of each clade. Many Neotropical sequences were unique, whereas North American sequences were frequently obtained from more than one host colony. For example, an identical ITS-sequence clustering within Clade B4 was obtained from 120 *T. septentrionalis* fungi, nine *M. turrifex* fungi, and three each from *T. arizonensis* fungi and *T. pomonae* fungi, essentially across the entire range of North American *Trachymyrmex* species from Arizona to New York.

Population structures of each fungal clade were not entirely consistent with recent expansion into the temperate zone. For example, Phylogroup 1 and Clades B2 and B3 did not show significant signatures of recent expansion (non-significant Tajima's *D* and Fu's F_S scores) for either tropical or North American population, although sample sizes of these groups are relatively low (Fig. 2, Table 1). On the other hand, the genetic structure of Clades B4 and B5 was consistent with recent expansion into North America (significant Tajima's *D* and Fu's F_S scores, Table 1). Surprisingly, Clade B5 also showed recent expansion in the tropics (Table 1).

While there was clear evidence of recent population expansion among *T. septentrionalis* fungi, which suggests a recent, post-Pleistocene range expansion of *T. septentrionalis* hosts (perhaps by Clade-B4-cultivating ants because these were the most common, Table 1, Fig. S2), evidence of recent expansion of fungi among other ant hosts was more equivocal. For example, even though our sample size of *M. turrifex* was considerably lower than that of *T. septentrionalis*, these fungi were scattered among Phylogroup 1 and clades B3–B5 (i.e., none were particularly abundant in any particular clade) (Table 2, Fig. S2). Fungi cultivated by species found in the southwestern U.S. (*T. arizonensis* and *T. pomonae*) were genetically very similar and all were nested within Clade B4. Tropical species were similarly variable; *P. cornetzi* and *M. zeteki* grow fungi from Phylogroup 1, Clades 2, 3 and 5, whereas *S. amabilis* and *S. mayri* appear to grow fungi only from Clade B5 (Fig. S2, Table 2) (De Fine Licht and Boomsma, 2014; Kooij et al., 2015a; Ješovnik et al., 2017).

3.4. Fungal diversity analyses

Rarefaction analysis of fungal lineages was hindered by high diversity and limited sampling among certain clade-region (Neotropics and North America) groupings and among certain host (ant species) groupings. For example, in some groupings, the Chao1 diversity estimator could not be calculated because there was only one individual colony per haplotype. That is, each of these individual colonies were growing a fungus that differed from fungi of other colonies by at least one base pair. As a result, for the clade-region groupings, we were only able to conduct rarefaction analysis and estimate our sampling effort among Neotropical Clade B3 and North American Clades B2, B4 and B5 (Fig. 2A–D). Other clade-region groupings either contained too few individual colonies to conduct rarefaction analyses, or all individual colonies cultivated fungi that were genetically distinct from one another. For the host groupings, all four North American host ant species (*T. arizonensis, T. pomonae, T. septentrionalis,* and *M. turrifex*) were growing a mixture of both genetically distinct and genetically similar fungi; whereas this was only true for one of the many Neotropical hosts, *M. zeteki* (Fig. 3A–E).

Despite tripling the sampling of Clade-B fungi to more than 300 samples, rarefaction analysis indicated that our sampling effort was inadequate to recover the entire spectrum of diversity of fungi grown by ant hosts. In general, Chao1 richness continually increased with the number of samples in each grouping (Figs. 2 and 3). These results also illustrate that, at the given number of individuals observed in each grouping, Chao1 richness was greater than our estimated richness. This indicates that not all of the fungal haplotypes in each clade or from each host have been captured from our sampling. For example, the analysis of our most heavily sampled North American fungal lineage (Clade B4, N = 43 unique sequences) indicated that there are probably 160 sequences that remain to be discovered (estimated total number is approximately 200 unique sequences; Fig. 2 C, Table 1). North American Clade B5 includes currently 17 known unique sequences, whereas there may be as many as 40 unique sequences that remain to be discovered (Fig. 2 D). Similarly, surveys of within-ant-host diversity were also quantitatively inadequate in *M. zeteki, T. septentrionalis*, and *M. turrifex*. For example, for *T. septentrionalis*, even though we recovered 50 unique sequences out of 180 screened for that ant

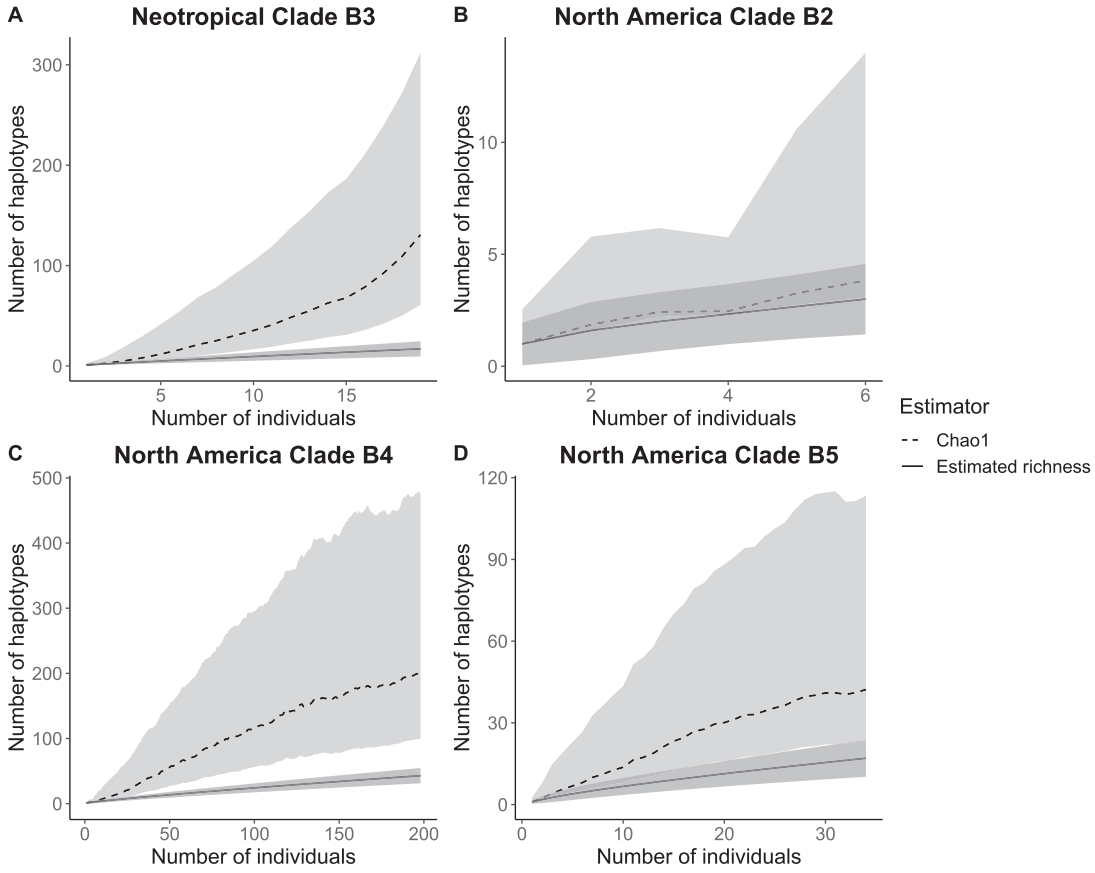

Fig. 2. Estimated haplotype richness by clade and region. Estimated fungal haplotype richness (with 95% confidence intervals), including Chao1 (broken line) and the estimated richness (S_{est}, solid line) calculated by EstimateS Version 9.1.0 (Colwell, 2013) after 100 randomizations without replacement. Samples are grouped by collection location and major fungal clade.

Table 1
Diversity of Clade B fungi grown by *Trachymyrmex*, *Sericomyrmex*, *Paratrachymyrmex*, and *Mycetomoellerius* ants. Sample size, number of unique haplotypes and population expansion (Tajima's *D* and Fu's F_s neutrality tests) of the five major groupings of Clade B fungi. Significant p values in bold ($\alpha < 0.05$).

	Phylogroup 1		Clade B2		Clade B3		Clade B4		Clade B5	
Population	North America	Neotropics	North America	Neotropics	North America	Neotropics	North America	Neotropics	North America	Neotropics
N	2	17	6	12	8	21	198	3	37	53
No. Unique Sequences	2	17	3	12	8	19	43	3	17	50
Tajima's D (p-value)	0	0.671 (0.790)	−1.337 (0.057)	0.224 (0.659)	−0.932 (0.190)	−0.516 (0.337)	**−1.781 (0.004)**	–	**−1.697 (0.012)**	**−1.33 (0.042)**
Fu's F_s	–	0.693 (0.474)	−1.458 (0.120)	−0.356 (0.249)	0.187 (0.224)	−2.01 (0.101)	**−25.22 (<0.0001)**	–	**−10.37 (0.001)**	**−6.78 (0.016)**

Table 2
Diversity of fungi grown by host ant species. Sample size, number of unique haplotypes and population expansion (Tajima's *D* and Fu's F_s neutrality tests) of fungi grown by *Trachymyrmex*, *Sericomyrmex*, *Paratrachymyrmex,* and *Mycetomoellerius* ants. Significant p values in bold ($\alpha < 0.05$). Undescribed hosts (e.g. '*Trachymyrmex* sp. 3 or C) or hosts with fewer than 5 records were not included.

	N	Fungal Clades	N. unique sequences	Tajima's D (p-value)	Fu's Fs
T. septentrionalis	**180**	**5**	**50**	**−1.908 (0.004)**	**−23.903 (0.001)**
M. turrifex	36	4	17	−0.489 (0.356)	**−5.481 (0.03)**
T. arizonensis	**28**	**1**	**10**	**0 (<0.0001)**	**−27.09 (<0.0001)**
T. pomonae	6	1	2	2.181 (0.99)	−2.242 (0.04)
M. zeteki	14	4	12	0.081 (0.549)	0.214 (0.33)
P. cornetzi	12	4	12	0.82 (0.821)	0.958 (0.42)
S. amabilis	19	1	19	**−1.946 (0.011)**	−1.132 (0.18)
S. mayri	9	1	9	**−2.073 (0.0001)**	1.682 (0.53)

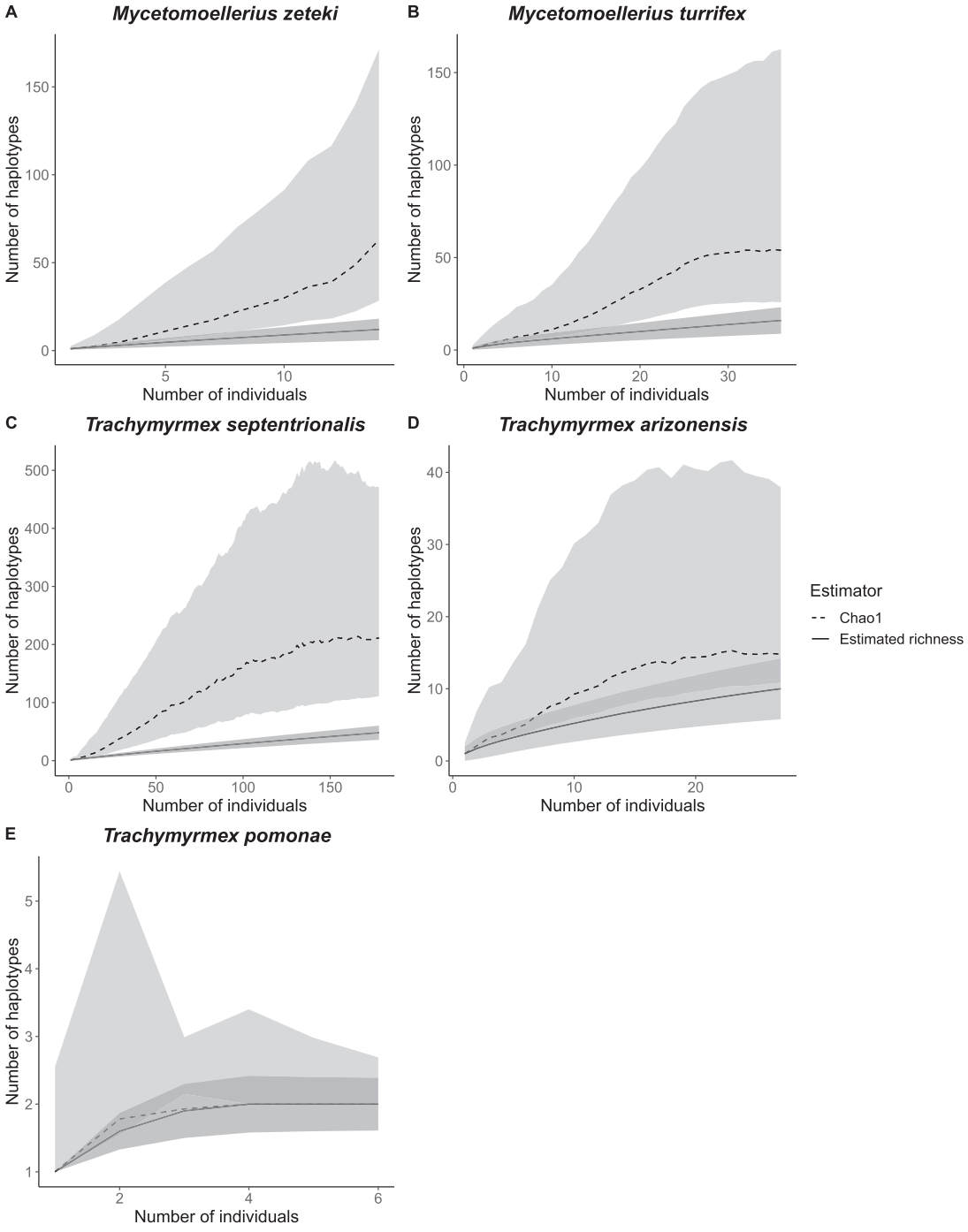

Fig. 3. Estimated haplotype richness by host. Estimated fungal haplotype richness (with 95% confidence intervals), including Chao1 (broken line) and the estimated richness (S_{est}, solid line) calculated by EstimateS Version 9.1.0 (Colwell, 2013) after 100 randomizations without replacement. Samples are grouped by ant host species.

host, extrapolations indicated that as many as 200 unique sequences remain to be discovered (Fig. 3 C). One exception may be fungi grown by *T. arizonensis*, which began to reach an asymptote and 95% confidence intervals overlapped. Groupings with fewer than seven individuals (e.g., North America Clade B2, North American *T. pomonae*) showed estimators converging/confidence intervals overlapping, but this was possibly an artifact of low sample size (Figs. 2 B, Fig. 3 E).

The diversity of fungi grown by North American *Trachymyrmex* and *Mycetomoellerius* ants was predicted by a range expansion model (recent expansion and reduced richness along range margins) to be lower than among fungi in the Neotropics. Analysis of fungal communities (all clades pooled into a single 'community') support this notion. Both Simpson and Shannon-Wiener indices indicated higher diversity in the Neotropics than North America [Simpson: 0.99 (Neotropics) vs. 0.70 (North America); Shannon-Wiener 4.62 (Neotropics) vs. 2.48 (North America)].

4. Discussion

The goal of this study was to evaluate the phylogenetic patterns and population structure of fungi grown by North American *Trachymyrmex* and *Mycetomoellerius* ants and compare these patterns to those of fungi associated with ant species in the Neotropics. Specifically, we tested whether North American Clade-B fungi (Mueller et al., 2018) were genetically depauperate as would be predicted by phylogeographic theory along range limits (Sexton et al., 2009; Mueller et al., 2011), which then could potentially relax host-symbiont specificity (Douglas, 2010; Kooij et al., 2015b). We discovered that, while genetic diversity of Clade-B fungi was slightly higher in the tropics than in North America, North America has been subject to multiple invasions by members of all major lineages of Clade-B fungi, so that the community of Clade-B fungi in North America is actually quite diverse. Diversity indices indicated relatively high genetic diversity in North America (*e.g.*, Simpsons index 0.7) was closer to 1 (infinite diversity) than to 0 (no diversity) probably because fungi from all major Clade B fungal lineages appear to have reached the range limit in the northern temperate zone. Moreover, some of these lineages appear to have expanded in North America (Clades B4 and B5) and in the tropics (Clade B5). While sampling intensity could produce a false test of population expansion (*e.g.*, by sampling highly similar individuals in close proximity), this is unlikely producing the current patterns because Clade B5 Neotropical fungi were collected from a large area (from southern Mexico to Brazil) from a variety of ant hosts (*Trachymyrmex, Mycetomoellerius, Paratrachymyrmex*, *Sericomyrmex*, *Acromyrmex* and *Atta*; Fig. S2). It seems, therefore, likely that Clade B5 fungi expanded recently throughout North and South America along with Clade B4 fungi in North America. Thus, the patterns observed may have less to do with the population-level processes along range limits than with overall synergisms among ant hosts and their fungal symbionts that produce the overall macroevolutionary patterns.

What do the results mean for the Fungal Control Model? Although population size in North America does not appear as diverse as populations in the Neotropics, there was measurable diversity among North American sequences. Thus, there would seemingly be enough variation within and among clades to select for fungal control. If fungi are surrounded by potential competitor fungi, there should be mechanisms to prevent the ants from adopting a novel fungal symbiont. Except for the southwestern *Trachymyrmex* species that primarily grow Clade B4 fungi (and at least some *T. arizonensis* Clade-A fungi), the southeastern species (*T. septentrionalis* and *M. turrifex*) grow fungi from at least 4 clades and exhibit notable within-clade diversity. While most *T. septentrionalis* colonies grow the same genotype of Clade B4 fungus (N = 120), there were 49 other unique ITS sequences grown by *T. septentrionalis* belonging to 2 other clades and Phylogroup 1 (Table 2, Fig S2). As a result, considering the within and among clade diversity typically grown by *T. septentrionalis*, a single genotype grown by a colony could have significant competitor fungi available in the population in neighboring colonies. Alternatively, fungal control might be relaxed in an environment containing considerable variation among fungal genotypes if colonies could not be expected to find them, especially if they are more frequently near other colonies growing the same fungal strain. However, the surveys in this study suggest that there is significant variation in the relative abundances of fungal clades in several sites in Texas. For example, at one of the localities sampled more frequently, the UT-Tyler campus forest (32.32° N, 95.25° W; an area of about 5 ha), we found fungi from Phylogroup 1 and Clades B3–B5. Clades B4 and B5 fungi were the majority cultivated by 12 and 9 colonies (50% and 38% respectively) while one colony cultivated a B3 fungus (4%) and two other colonies grew fungi from Phylogroup 1 (8%; Fig. S2). It would thus appear that colonies growing one type of fungus could be exposed to a very different fungal strain than they are currently growing. On the other hand, another heavily sampled locality, UT-Austin's Stengl Lost Pines Biological Station (30.09° N, 97.17°W; a 230 ha property), showed a very different pattern with 47 colonies (92%) growing Clade B4 fungi, three colonies growing Clade B3 (6%) and a single colony (2%) growing Clade B5. The proximate mechanisms determining regional and local fungal relative abundances are unknown. More systematic (rather than opportunistic) surveys could determine the relative abundance and how frequently a colony could encounter a different fungal strain.

One surprise in this study was that Clade B4 and B5 fungi largely dominate the fungal symbiont fauna in North America. One explanation may be that southern North America has many subtropical if not temperate characteristics and these clades may be better adapted to such conditions than Phylogroup1, Clade B2 and B3. For example, seasonal conditions may range from dry deserts, cold winters with periodic (if not extended) sub-freezing conditions, to conditions more typical of the humid tropics in terms of temperature and rainfall (Noss et al., 2015). However, Phylogroup 1, Clades B2 and B3 also appear relatively rare in the tropics compared to Clade B5, for example. Unfortunately, with the current dataset we cannot rule out the role of climate. Only Clades B4 and B5 were found relatively far north; Norman, Oklahoma - 35°N; and some Clade B5 fungi have been found in northeastern Argentina - approximately 27°S (Mueller et al., 2018), in addition to the tropics proper (French Guiana, Peru, Panama; Fig. S2). Interestingly, Clades B4 and B5 – two crown group radiations – appear to be the most abundant Clade-B fungi, which suggests that there may be unique attributes of these fungi in a variety of environments and hosts. Similarly, Phylogroup 1, Clades B2 and B3 could be at a disadvantage in some unknown manner; however, the neutrality tests (Tajima's *D* and Fu's F_s) do not support range contractions (significant positive values) that could result if genotypes are in an unfavorable environment (Tables 1 and 2). Another alternate explanation may be that Clades B4 and B5 fungi have radiated fairly recently and are currently sweeping through ant populations at the expense of more ancestrally diverging lineages that are no longer favored for unknown reasons. For example, crown ages of leaf-cutting ants (*Atta* and *Acromyrmex*) suggest they diverged from *Trachymyrmex*-like ancestors much earlier than Clade-A fungi (*L. gongylophorus*) diverged from Clade-B fungi, with leaf-cutting ants having adopted Clade-A fungi relatively recently (Mikheyev et al., 2010; Nygaard et al., 2016). On the other hand, stem ages suggest that Clade-A fungi may have arisen before leaf-cutting ants, which may explain why some *Trachymyrmex* cultivate Clade-A fungi (Mueller et al., 2017, 2018). Unfortunately, understanding the divergence dates of fungi and its role in specificity will require robust fungal genomes, which have not been sequenced due to the highly polyploid nature of higher attine fungi (Kooij et al., 2015a; Nygaard et al., 2016). Additionally, cross-fostering experiments could help determine the synergistic basis, if any, of many of these naturally occurring host-symbiont pairings (Mehdiabadi et al., 2006; Seal and Tschinkel, 2007; Seal and Mueller, 2014; Seal et al., 2014; DeMilto et al., 2017).

More would be gained by more thorough surveys in the tropics, e.g., tropical South America and in North America, such as the southeastern US (Florida) and the southwest US (Arizona) (Rabeling et al., 2007; Mikheyev et al., 2008; Seal et al., 2015). The new collections in this study were somewhat opportunistic; samples were collected when colonies were encountered until 3–10 colonies were obtained from a single site. For instance, most members of Phylogroup 1 were tropical except for two specimens collected on the UT-Tyler campus (Texas). Similarly, the vast

majority of (N = 198) of specimens clustering in Clade B4 were collected in North America (from Arizona to New York), except for two specimens collected in northeastern and southeastern Brazil (Ješovnik et al., 2017) and a third specimen clustering with these sequences grown by an *A. striatus* colony in northeastern Argentina (Mueller et al., 2018). Whether this reflects natural abundances of these taxa or is a sampling artifact is unknown. While diversity does appear to be higher in the Neotropics, it is important to note that the total area surveyed in North America is noticeably smaller than what is considered Neotropical in this study (Mexico to Argentina). Thus, a good part of the Neotropics might be undersampled and total area may be a confounding variable (Colwell and Lees, 2000).

Despite collecting 225 samples (of the 358 total samples used in the analyses) over the course of three years in part of the known range of *T. septentrionalis*, the most widely sampled species (N = 180) (Table 2), the rarefaction analyses suggest that we are nowhere near recovering the total diversity of ITS sequences predicted for fungi of *T. septentrionalis*. The single exception where sampling effort seemed adequate were sequences of *T. arizonensis* and perhaps *T. pomonae*. However, this might be biased because all of these samples came from the Southwest Research Station in a single canyon in the Chiricahua Mountains of southeastern Arizona. Thus, future intensive surveys in small regions may not be the optimal strategy to maximize collection of diverse fungi from an ant host. Rather, other canyons or mountain ranges altogether should be widely surveyed. Because *T. arizonensis* is known to occur in mid-elevation 'Sky Islands' (isolated mountain ranges separated from the southern Rocky Mountains by lowland deserts) from west Texas to western Arizona (Rabeling et al., 2007), it is highly likely that fungal diversity in *T. arizonensis* and *T. pomonae* has so far been undersampled. Indeed, there are recent reports of one population of *T. arizonensis* colonies growing both Clade-B and Clade-A fungi in southeastern Arizona (Mueller et al., 2018).

Clade-B fungi appear to be quite mobile by dispersing somewhat independently of their hosts, even though it is unclear how this might happen since no higher attine symbionts have ever been found outside of ant nests, unlike the so-called 'lower Attina' whose symbionts appear very similar to free-living fungi (Vo et al., 2009). Nevertheless, the potential for independent dispersal of Clade-B symbionts appears very similar to conclusions drawn by co-phylogenetic analyses of Clade-A fungi and their hosts (Mueller et al., 2017, 2018; Smith et al., 2019). For example, the Texas leaf-cutting ant *A. texana* appears to grow a mixture of Clade-A fungi of temperate and tropical origin across its range (Mueller et al., 2011). Similarly, the major Clade-B fungal clades are grown by many ant species, including *Mycetomoellerius*, *Paratrachymyrmex* and *Trachymyrmex* sensu stricto (Solomon et al., 2019) While *T. septentrionalis* grows fungi from all five Clade-B subclades and *M. turrifex* from four, some identical fungal sequences were observed to be shared among North American ants. For example, two sequences of Clade B4 fungi collected in Norman, Oklahoma were also found grown by *T. arizonensis* colonies in Arizona (approximately 1144 km linear distance). Similarly, an identical Clade B4 fungus was found grown by colonies of *T. arizonensis* (N = 3), *T. pomonae* (N = 3), *T. septentrionalis* (N = 120) and *M. turrifex* (N = 9). This particular genotype was found grown by colonies in Florida, Arkansas, Illinois, Texas and Arizona (or a linear distance of 2368 km between Tallahassee, Florida and Portal, Arizona). This genotype was very similar to the fungus grown by three specimens collected in Brazil and Argentina (GenBank sequences **JX259063**, **JX259067**, and **KT898383**) – the differences were only among 4 ambiguous base calls. Future challenges would be to include comprehensive samples from a larger portion of lowland Neotropics, especially Brazil, but also to develop more powerful markers (e.g., genotyping-by-sequencing) to evaluate the dispersal ability of symbionts and to understand how symbiont biology may have changed as ant-fungus associations dispersed into regions outside the tropics.

This study demonstrates that the diversity of Clade-B fungi cultivated by lineages of *Trachymyrmex* sensu lato is likely quite immense, even at and near the range margins in North America. This study also calls into question whether the biology of range limits play a large role in symbiont diversity and specificity, generally. For example, corals located along tropical-temperate transitions did not show relaxed specificity at higher latitudes with *Symbiodinium* symbionts (LaJeunesse et al., 2008; De Palmas et al., 2015) or contain low genetic diversity (Wicks et al., 2010). Similarly, *Symbiodinium* diversity was not impacted by depth (distance from sunlight) in a sea fan (Forcioli et al., 2011). Likewise, specificity of ectomycorrhizal fungi was not impacted along range margins of host tree species (Lankau et al., 2015), even though ectomycorrhizal fungal richness was much lower (Lankau and Keymer, 2016). As a result, at least at continental scales, some symbioses might be particularly genetically diverse and perhaps robust to environmental heterogeneity and climate change.

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Supplementary data

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