Arbuscular mycorrhizal fungal community composition is altered by long-term litter removal but not litter addition in a lowland tropical forest

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SUMMARY

- Tropical forest productivity is sustained by the cycling of nutrients through decomposing organic matter. Arbuscular mycorrhizal (AM) fungi play a key role in the nutrition of tropical trees, yet there has been little experimental investigation into the role of AM fungi in nutrient cycling via decomposing organic material in tropical forests.

- We evaluated the responses of AM fungi in a long-term leaf litter addition and removal experiment in a tropical forest in Panama. We described AM fungal communities using 454-pyrosequencing, quantified the proportion of root length colonised by AM fungi using microscopy, and estimated AM fungal biomass using a lipid biomarker.

- AM fungal community composition was altered by litter removal but not litter addition. Root colonisation was substantially greater in the superficial organic layer compared to the mineral soil. Overall colonisation was lower in the litter removal treatment, which lacked an organic layer. There was no effect of litter manipulation on the concentration of the AM fungal lipid biomarker in the mineral soil.

- We hypothesise that reductions in organic matter brought about by litter removal may lead to AM fungi obtaining nutrients from recalcitrant organic or mineral sources in the soil, besides increasing fungal competition for progressively limited resources.

INTRODUCTION

The productivity of most tropical forests is sustained by symbiotic associations between plants and arbuscular mycorrhizal (AM) fungi (Read, 1991; Alexander & Lee, 2005). AM fungi play crucial roles in nutrient cycling and are also major vectors of carbon (C) in the global C cycle (Johnson *et al.*, 2013). AM fungi obtain up to 20-30% of total plant

photosynthates (Drigo *et al.*, 2010) and may enhance the decomposition of organic matter, releasing substantial quantities of CO_2 to the atmosphere through their respiration (Nottingham *et al.*, 2010).

Tropical forest growth currently constitutes the largest terrestrial sink for anthropogenic CO_2 (Oren *et al.*, 2001) and thus makes a substantial contribution to the regulation of the global climate system (Field *et al.*, 1998). Anticipating future effects of anthropogenic change on tropical forests demands a clearer understanding of how nutrient availability limits forest productivity, and the roles of AM fungi in complex scenarios of nutrient limitation and co-limitation. Nonetheless, AM fungi are under-investigated in tropical systems in general, and tropical forests in particular (Alexander & Selosse, 2009).

It is widely hypothesised that the symbiotic function of AM fungi is determined by the relative availability of C, nitrogen (N), and phosphorus (P; Johnson, 2010; Johnson *et al.,* 2013). This is based on evidence which shows that fertilisation with N and P can reduce AM fungal colonisation of roots (Johnson *et al.*, 2003), and that the relative amounts of N and P determine mycorrhizal symbiotic function (Johnson, 2010). In some cases this may cause AM fungi to behave less mutualistically (Johnson, 1993); where neither N or P is limited, the only limitation to fungal growth is the supply of plant C, meaning that fungal C demand can increase to the point where plant growth is depressed (Johnson, 2010).

Much current understanding concerning the function of AM fungal symbioses comes from studies that explore how variation in nutrient availability affects AM fungal characteristics (eg. Treseder, 2004; Wurzburger & Wright, 2015). Amongst these, nutrient addition experiments are one of the most widely used approaches, particularly in field settings (Treseder, 2004). Nutrient addition is hypothesised to affect AM fungi either directly, by alleviating fungal nutrient limitation and thereby stimulating fungal growth (Treseder & Allen, 2002), or indirectly, by causing plants to reduce investment of carbohydrate in their AM fungal partners (Mosse & Phillips, 1971; Johnson, 2010).

Besides altering AM fungal biomass, nutrient addition may affect AM fungal community composition and diversity. Changes in community composition and diversity are likely to arise from differences in the functional properties of AM fungal taxa and their ability to compete with other fungi (AM or saprobe) for key resources (Hart & Reader, 2002; Maherali

& Klironomos, 2007; Powell *et al*., 2009). For instance, different AM fungal taxa can vary in the translocation of P (Ravnskov & Jakobsen, 1995) or N (Veresoglou *et al.,* 2012) to plant partners, carbon storage and demand (Pearson & Jakobsen, 1993), relative allocation to intra- and extra-radical biomass (Hart & Reader, 2002), and growth and life-history strategy (Hart & Reader, 2002; Maherali & Klironomos, 2007; Powell *et al.*, 2009). Furthermore, plant-AM fungal combinations perform differently in alternative settings, with wide range of symbiotic outcomes (Klironomos, 2003; Powell *et al.*, 2009). Consequently, the advantage of AM fungal associations will vary according to the prevailing conditions and the ecological niche of the fungal partner. Evaluation of community parameters thus provide important information to supplement the aggregate metrics of root colonisation and concentration of the AM fungal biomarker lipid (a proxy for AM fungal biomass), which cannot distinguish between members of the AM fungal community.

In addition, AM fungal species that share a common evolutionary history may also share traits and ecological functions (Maherali & Klironomos, 2007; Powell *et al.*, 2009), and community data can thus be used to infer the ecological processes structuring AM fungal communities. Phylogenetically over-dispersed communities (communities consisting of taxa that are less related to each other than expected by chance) are hypothesised to be structured by competition, preventing closely related and functionally similar taxa (those sharing a common niche) from co-occuring. By contrast, phylogenetically under-dispersed (or clustered) communities are hypothesised to be structured by habitat filters; features of the environment that permit only the co-occurence of species with specific traits or ecological tolerances, and which can cause taxa with similar traits to respond in similar ways to environmental pressures (Webb *et al.*, 2002; Maherali & Klironomos, 2007).

The great majority of nutrient addition studies apply inorganic fertilisers (eg. see Treseder, 2004). These studies are useful in highlighting the roles of individual nutrients and simulating the effects of inorganic nutrient deposition. However, fertilisation treatments are artificial and do not mimic pathways of nutrient cycling under natural conditions (Sayer & Banin, 2016). Furthermore, the regulation of plant-AM fungal relations is strongly dependent on the relative availability of different nutrients (Treseder & Allen, 2002; Johnson, 2010), whereas the addition of large quantities of one or more inorganic nutrients (e.g. N, P, K) strongly distorts stoichiometric relationships, and largely neglects the role of organic matter in nutrient cycling (Sayer & Banin, 2016).

Under natural conditions, nutrient cycling in forests occurs largely through litterfall, root death, root exudates, decomposition, and the growth and death of microorganisms (Attiwill & Adams, 1993; Leff *et al.*, 2012). It is via these processes that the regulatory processes governing plant-AM fungal exchange have evolved. Indeed, over large latitudinal gradients there is a strong relationship between leaf litter quality, the organic matter resulting from its degradation, and the predominant mycorrhizal type in a given bioregion (Read, 1991). Nonetheless, there have been few experimental investigations into the effects of leaf litter amendments on AM fungi in highly diverse tropical forests.

Although multiple lines of evidence suggest a key role for AM fungi in cycling nutrients via organic sources, the majority of studies investigating the effects of organic amendments on AM fungi have been conducted in experimental microcosms, and most have examined changes in biomass rather than community parameters (Hodge, 2014). These experiments demonstrate that AM fungal hyphae preferentially proliferate in organic substrates in experimental microcosms (Hodge & Fitter, 2010), are able to capture N from organic substrates (Leigh *et al.*, 2009), and can enhance the decomposition of organic material (Hodge, 2014). The few existing field studies show that organic matter additions in agricultural systems tend to increase AM fungal colonisation of plant roots and hyphal abundance in soils (Gryndler *et al.*, 2005; Gosling *et al.*, 2010). Furthermore, AM fungal hyphae can grow into decomposing leaf litter on tropical forest floors (Herrera *et al.*, 1978; Posada *et al.*, 2012; Camenzind & Rillig, 2013). Together, these studies strongly suggest that AM fungal hyphae are important in recycling nutrients from leaf litter. This is likely due to tightly coupled interactions between AM fungi and saprophytic fungi and bacteria (Herman *et al.*, 2012) given that AM fungi have not been shown to possess saprophytic capabilities (Hodge, 2014).

We investigated AM fungal responses to altered organic matter inputs in a lowland tropical forest in Panama using an existing long-term litter manipulation experiment in which nine years of litter removal and addition treatments have altered fine root biomass (Sayer *et al.*, 2006a), litter production, foliar and litter nutrient concentrations, and soil nutrient pools (Vincent *et al.*, 2010; Sayer & Tanner, 2010b). This platform provided a unique opportunity to evaluate the responses of AM fungal communities to changes in organic matter inputs in a well-studied lowland tropical forest setting.

We hypothesised that: i) litter addition would increase net AM fungal abundance, given the well-documented stimulatory effects of organic matter additions on AM fungal growth, ii) litter removal would also increase net AM fungal abundance, given that plants may increase investment in AM fungi when nutrient availability is reduced (Johnson, 2010), iii) that the addition or removal of organic matter would result in changes in the AM fungal community composition, and iv) that litter manipulation would alter the ecological processes structuring AM fungal communities, and that this would be reflected in changes in the degree of relatedness (or phylogenetic structure), of AM fungal communities.

MATERIALS AND METHODS

Site description and experimental design

The Gigante Litter Manipulation Experiment (GLMP) is located on the Gigante Peninsula ($9^{o}06'$ N, $79^{o}54'$ W) within the Barro Colorado Nature Monument (BCNM) in Panama, Central America. Nearby Barro Colorado Island (BCI; c. 5 km from the study site) has a mean annual rainfall of 2600 mm, with a strong dry season between January and April and a mean annual temperature of 27 ^{o}C (Leigh, 1999). Tree species composition and canopy height are characteristic of mature (>200 year old) secondary forest (Wright *et al.*, 2011) and the soils are classed as moderately acidic Oxisols (Dieter *et al.*, 2010; Turner & Wright, 2013), with low concentrations of available P and moderate concentrations of base cations (Turner et al., 2013). The GLMP consists of fifteen 45 m × 45 m plots; starting in 2003, leaf litter from five plots was raked up once a month (litter removal treatment; L-), immediately added to five plots where it was distributed as evenly as possible (litter addition treatment; L+), and five plots were left undisturbed as controls (C; see Sayer & Tanner 2010 for details).

Sampling

In May 2012, after nine years of treatments, we sampled at six points in the inner 30 m x 30 m of each of the 15 experimental plots (a total of 30 samples per treatment); we selected sampling points at random using random number sheets to delineate point coordinates, with the provision that all points were separated by at least 3 m. At each sampling point, we collected the litter (Oi) and fermentation (Oe) horizons from a 78.5 cm^2 area, using a knife to cut around the edge of a metal disk (C and L+ treatments only; the L- treatment lacked an

organic horizon), and two cores from the mineral soil (0-10 cm depth) using a 5-cm diameter corer (all treatments). To prevent cross-contamination, we wiped down and flame-sterilised all equipment in between samples, handled all samples with fresh latex gloves, and double-bagged samples in sealed Ziploc^{TM} bags. All samples were stored at 4^{o}C and processed within 36 hours of returning from the field. Root samples were obtained from one of the two cores per sampling point by washing away soil and organic matter under a continuous stream of filtered water over a sieve with a mesh size of 500 μm. We retained fine roots (≤ 1 mm in diameter) for further analysis, drying a subsample over silica gel for DNA extraction, and storing a second subsample in 70% ethanol for microscopic analysis. The remaining soil cores were sieved to remove stones and roots, composited to make one sample per plot, and thoroughly homogenised. 20 g subsamples for lipid analysis were frozen at -80^{o}C for 12 h, lyophilised, and stored dry at -80^{o}C until further processing.

Prior to lipid and nutrient extractions, an equal mass of each sample was pooled to make one composite sample per plot (a total of 15 samples). Prior to DNA extraction, the six root samples per plot were individually pulverised in a homogeniser (TissueLyser II, Qiagen), and an equal mass of each sample was pooled to make one composite sample per plot (a total of 15 samples). Microscopic analysis of root samples was performed on individual samples (total = 6 samples per plot, 90 samples in total).

AM fungal abundance

We used the percentage of root length colonised as a measure of intra-radical AM fungal abundance (McGonigle *et al.*, 1990). We soaked and rinsed the root samples with distilled water to remove the ethanol. Roots were then cleared by autoclaving in 5% KOH for 5-60 minutes; bleached in solution of ammonia in 3% H_2O_2 for 15-60; acidified in 2% HCl for 30 minutes; and stained with 0.05% trypan blue (in a 1:1:1 solution of distilled water, glycerol and lactic acid) for 20 minutes at 60^{o}C. The optimum clearing and bleaching time varied depending on the thickness and pigmentation of the roots. We quantified AM fungal colonisation by hyphae, vesicles and arbuscules using a compound light microscope at 200× magnification, according to the method of McGonigle et al. 1990 with at least 100 intersections per sample, and one sample per core. AM fungal colonisation was expressed as the percentage fine root length colonised by AM fungal hyphae, vesicles or arbuscules.

We used the neutral lipid fatty acid (NLFA) 16:1ω5 as a biomarker for extra-radical AM fungal biomass. We performed lipid extraction and analysis according to Frostegård et al. (1993) with modifications (Nilsson *et al.*, 2007). Briefly, lipids extracted from 4 g lyophilised soil per plot were fractionated into neutral lipids, glycolipids, and polar lipids on silica columns by successive elution with chloroform, acetone and methanol. Methyl nonadecanoate (FAME 19:0) was added as an internal standard, and neutral and polar fractions were converted to fatty acid methyl esters (FAMEs) prior to analysis on a gas chromatograph with a flame ionisation detector and a 50 m HP5 capillary column (Hewlett Packard, Wilmington, DE, USA). The mean NLFA to PLFA ratio across all samples was 1.3, suggesting that NLFA 16:1ω5 is an effective AM fungal biomarker in these soils (Olsson, 1999).

Soil chemistry

Measurement of inorganic N (NO_3^- and NH_4^+), resin-extractable P, organic P, and pH was performed as described in Turner et al. (2013). Analysis of total N and C was performed on air-dried soils by automated combustion and gas chromatography on a Thermo Flash EA1112 analyzer (CE Elantech, New Jersey, USA). Organic P was extracted in a mixture of 0.25 M NaOH and 0.05 M EDTA, and analysed as described by Turner et al. 2008. Exchangeable cations were extracted in 0.1 M $BaCl_2$, with detection by ICP-OES (Hendershot et al. 2008), and effective base saturation (EBS) was calculated by dividing the cmol of positive charge per kg dry soil of exchangeable bases (Ca + K + Mg + Na) by that of the total cations (Al + Ca + Fe + K + Mg + Mn + Na; Hendershot et al. 2008).

DNA extraction and sequencing

We extracted DNA from 50 mg of pulverised root using MoBio PowerPlant DNA isolation kits according to the manufacturer's instructions (MoBio Laboratories Inc., Carlsbad, CA, USA).

We amplified the partial small subunit (SSU) region of 18S ribosomal DNA (*c.* 550 bp) with the universal eukaryotic primer NS31 (Simon *et al.*, 1992) and the AM fungal-specific primer AM1, which amplifies the major families of the Glomeromycota (Helgason *et al.*, 1998). We chose this primer set because it is widely represented in sequence databases, and because we wanted to facilitate comparisons with previous work using these primers. In addition, these primers have been demonstrated to have extremely low PCR bias against artificially

assembled community templates (Cotton *et al.*, 2014). Prior to amplification, the primers were modified by the addition of the 454 pyrosequencing adaptors A and B, in addition to a 10 bp multiplex identifier (MID) on the forward primer (NS31). We conducted duplicate polymerase chain reactions (PCRs) in 25 µl sample volume using Phire hot start II DNA polymerase (Life Technologies LTD, Paisley, UK). Conditions were: 98^{o}C for 1 minute; 32 cycles of 98^{o}C for 10 s and 72^{o}C for 15 s; and a final extension phase of 72^{o}C for 2 minutes.

We gel-purified the PCR products using MinElute PCR purification kits (Qiagen Ltd, West Sussex, UK) and pooled the samples in equimolar concentrations, evaluating the concentration of DNA in the cleaned PCR products using Quant-iT PicoGreen dsDNA Assay Kit (Invitrogen, Life Technologies LTD, Paisley, UK). Amplicon libraries were distributed on PicoTiter Plates and sequenced on an FLX Titanium system using Lib-L shotgun chemistry (Roche, Basel, Switzerland). No sequences were detected in the blanks included as negative controls at each of the extraction, PCR, gel purification, and quantification steps.

Bioinformatic analysis

All bioinformatic analyses were performed using the software mothur (Schloss *et al.*, 2009) unless otherwise stated. Sequence filtering was performed with the sff.multiple quality filtering protocol. Reads were removed from the dataset if they did not contain the 10 bp MID, had > 1 error in the barcode sequence, > 2 errors in the forward primer, or were shorter than 200 bp in length. After quality filtering and removal of barcode and primer sequences, clustering was performed using the algorithm Clustering 16S rRNA for Operational Taxonomic Unit (OTU) Prediction (CROP), an unsupervised Bayesian clustering method that forms clusters based on the organisation of sequences without setting a hard similarity cutoff (Hao *et al.*, 2011). To provide finer taxonomic resolution, we set the *i* and *u* parameters to 2% cluster difference rather than the conventional 3% because the SSU region has relatively low variation (Öpik *et al.*, 2013; Davison *et al.*, 2015). The centre sequence from each cluster was used as a representative sequence in subsequent analyses.

Sequence alignment was performed with the software MAFFT v7.149b (Katoh *et al.*, 2002) using the L-INS-i algorithm (iterative refinement using local pairwise alignment) and the alignment from Krüger et al. (2012) as a backbone. Alignments were improved with MUSCLE (Edgar, 2004) using the –refine option. Trees were built using RAxML v. 8.0

(Stamatakis, 2014) with GTR GAMMA implementation, and bootstrap values based on 1000 runs.

We used the Basic Local Alignment Search Tool (BLAST, Altschul *et al.*, 1990; minimum e-value 10^{-30}) on one representative sequence from each cluster iteratively against three databases in the following order of preference: i) sequences from Krüger et al. (2012); ii) all virtual taxa (VT) from the MaarjAM AM fungal sequence database (www.maarjam.botany.ut.ee); and iii) all 18S Glomeromycotan sequences from SILVA database. Non-Glomeromycotan clusters were removed when the highest blast match did not correspond to an AM fungal sequence in any of the three datasets.

Clusters were named based on matches to database entries at > 97% covering a minimum of 80% of the query sequence. We used the generic names from Krüger et al. (2012), and VT numbers from the MaarjAM database. Where clusters did not match a VT at > 97% we assigned a name based on the highest VT match and phylogeny (eg. Glomus_OTU1). We fused clusters based on matches to database sequences > 97% and the tree topology obtained from RaXML. Clusters that occurred in < 2 samples, and with < 5 reads total were removed from the dataset. Raw sequence data were deposited in the International Nucleotide Sequence Database Sequence Read Archive (accession no. SRP076949).

Statistical analysis

All statistical analyses were conducted in R version 3.1.2 (R Development Core Team, 2014).

Multivariate analysis of AM fungal communities

We accounted for variation in the number of sequences between samples by using a variance stabilising (VS) transformation of the OTU table, implemented with the DESeq2 package (Love *et al.*, 2014), according to McMurdie and Holmes (2014). This approach avoids the need for rarefying, which can result in data that misrepresent the original community (McMurdie & Holmes, 2014). All subsequent analysis was performed on the VS transformed OTU table, using the copy number of DNA sequences as a measure of relative abundance of each OTU.

To examine the effect of litter manipulation on AM fungal community composition, we used multivariate generalised linear models (M-GLMs) with negative binomial error structures using the mvabund package (Wang *et al.*, 2012). M-GLMs provide a more robust way to analyse multivariate community data than do distance-based approaches such as PERMANOVA (Warton *et al.*, 2015). We ascertained the degree to which individual OTUs were affected by litter manipulation using DESeq2 (Anders & Huber, 2010), which estimates the effect size (as logarithmic fold change) and reports *P*-values adjusted for multiple comparisons.

To visualise differences in AM fungal communities across litter manipulation treatments we used non-metric multidimensional scaling (NMDS) ordination, using the metaMDS function in the vegan package (Anderson 2001, Oksanen et al. 2010). Ordination was based on Bray-Curtis dissimilarity calculated from square-root transformed abundances. The range of data values was large, and a square root transformation was applied to improve the quality of the ordination by reducing the weighting of the most abundant OTUs (Legendre & Legendre, 2012; Oksanen *et al.*, 2010).

Soil physical characteristics were standardised to zero mean and unit variance, and fit to the NMDS ordinations (function envfit from the vegan package) with significance ascertained using 9999 permutations. Individual values of exchangeable cations were collapsed into the metric of effective base saturation (EBS). Organic phosphorus correlated closely with resin-extractable phosphorus ($r^2 > 0.7$) and was omitted, since resin-extractable phosphorus better approximates the plant-available phosphorus fraction (Condit *et al.*, 2013).

Community phylogenetic structure

We asked whether litter manipulation altered the degree of relatedness between taxa in AM fungal communities. We used two indices of community phylogenetic structure: Net Relatedness Index (NRI) and Nearest Taxa Index (NTI; (Webb, 2000). Positive values of these metrics indicate that taxa in a community are on average more closely related to each other than to members of the regional taxon pool (phylogenetically clustered), and negative values indicate that taxa in a community are less closely related (phylogenetically over-dispersed). NRI is sensitive to tree-wide phylogenetic patterns, and NTI is sensitive to phylogenetic community patterns close to the tips of the phylogeny. Observed values of these metrics were compared to 10,000 null communities generated using the 'independentswap'

algorithm, which maintains column and row totals and accounts for differences in community richness and taxon prevalence (Gotelli, 2000). Statistical significance of phylogenetic structure was ascertained using a two-tailed *t*-test. Community phylogenetic analysis was performed using the picante package (Kembel & Ackerly, 2010).

Univariate analysis of AM fungal abundance and diversity, and soil physical characteristics

We analysed the effects of litter manipulation on the concentrations of NLFA 16:1ω5 in the soil, AM fungal colonisation of plant roots, AM fungal OTU richness and predominance, and metrics of phylogenetic community structure (NRI and NTI) using linear models having confirmed that all variables met the assumptions. Where the main effect of litter manipulation was significant, we performed Dunnett's *post-hoc* analysis to compare each treatment with the controls.

To ascertain whether AM fungal colonisation of roots was greater in the mineral soil or organic layer we built linear mixed effects models (using the lme4 package; Bates et al. 2014). Models included 'layer' and 'treatment' as fixed effects, and 'plot' as a random effect. The significance of fixed effects was assessed by comparing nested models using parametric bootstrapping with 10000 simulations, using the PBmodcomp function from the pbkrtest package (Halekoh & Højsgaard, 2014). Results are reported as significant at $\alpha < 0.05$.

RESULTS

Soil chemistry

Soil nutrients were lower in litter removal compared to litter addition treatments for inorganic N; resin and organic P, pH, and extractable Ca, Mg, and Mn (K was not significantly lower). Compared to the controls, the soils in the L- plots had lower concentrations of inorganic N, resin and organic P, Ca, and Mg, whereas soils in the L+ plots had higher concentrations of resin P, and Ca (Figure 1, Table S1). A full discussion of the effects of litter manipulation on soil chemistry is provided in Sayer & Tanner (2010) and Sayer *et al*. (2012).

AM fungal abundance

There was no significant effect of litter manipulation on the proportion of root length colonised by any AM fungal structure in the mineral soil (total colonization, hyphae, vesicles or arbuscules), although for each of the structures there was a trend towards higher root

406 colonisation in both litter removal and litter addition treatments compared to the controls
407 (Figure 2; total colonisation: $F_{2,12} = 1.7$, $P = 0.23$; hyphae: $F_{2,12} = 1.4$, $P = 0.29$; vesicles: $F_{2,12}$
408 $= 2.5$, $P = 0.13$; arbuscules: $F_{2,12} = 1.3$, $P = 0.31$). In the control and litter addition plots, the
409 proportion of root length colonised by all AM fungal structures was substantially greater in
410 the superficial organic layer than in the mineral soil (significant 'layer' term; hyphae:
411 likelihood-ratio test (LRT) = 50.0 $P < 0.001$; vesicles: LRT = 19.6, $P < 0.001$; arbuscules:
412 LRT = 28.6, $P < 0.001$; all structures: LRT = 51.6, $P < 0.001$; Figure 2). Because root
413 colonization was highest in the superficial organic layer, the overall abundance of AM fungi
414 was lower in the litter removal treatment, which lacked this layer.
415
416 There was no effect of litter manipulation on AM fungal biomass in the mineral soil
417 (concentration of NLFA 16:1ω5; Figure S1), nor was AM fungal biomass correlated with any
418 of the measured soil variables (soil pH: $F_{1,13} < 0.001$, $P = 0.98$; effective base saturation: $F_{1,13}$
419 $= 0.01$, $P = 0.92$, resin-extractable phosphorus: $F_{1,13} = 0.12$, $P = 0.74$; and inorganic nitrogen:
420 $F_{1,13} = 0.54$, $P = 0.48$).
421
422 **AM fungal community composition and structure**
423 Four AM fungal families were represented in the sequencing dataset (Acaulosporaceae,
424 Archaeosporaceae, Gigasporaceae, Glomeraceae; Figure 3), indicating reasonable taxonomic
425 coverage of the Glomeromycota (based on the classification of Redecker *et al.* 2013). No
426 members of the Diversisporaceae, Paraglomeraceae, Geosiphonaceae, Ambisporaceae,
427 Claroideosporaceae or Pacisporaceae were detected. Rarefaction curves for each sample
428 indicated that sequencing intensity was sufficiently high to detect the majority of OTUs.
429 Rarefaction curves pooled by experimental treatment approached asymptotes, indicating that
430 sampling effort was sufficient to capture the range of AM fungal taxa across the sites (Figure
431 S2). A total of 10,197 sequences were retained after quality control, clustered into 72 OTUs,
432 and 95.9% of all sequences matched Glomeromycota in the databases. Fifty-six OTUs
433 remained after blasting, filtering, merging, and trimming (exclusion of OTUs with a total of 5
434 or less reads), representing a total of 8825 sequences. Each sample (1 per plot) contained a
435 mean of 18 OTUs (range: 11-24), and the mean number of sequences per sample was 588
436 (range: 237-1225; Table S2). A phylogenetic tree is provided in Figure S3.
437
438 Overall AM fungal community composition was altered by litter removal but was not
439 significantly affected by litter addition (Multivariate GLM: Wald $_{2,12} = 11.5$, $P < 0.003$;

treatment contrast for litter removal: Wald = 9.2, $P < 0.003$ and for litter addition: Wald = 5.9, $P = 0.24$; Figure 4). There were no significant differences among treatments when the analysis was repeated at levels of genus and family (multivariate GLM; genus: Wald $_{2,12}$ = 3.9, $P = 0.24$; family: Wald $_{2,12} = 2.1$, $P = 0.66$; Figure 3). In the analysis of individual OTUs (using the DESeq2 package), litter removal significantly ($P < 0.05$) reduced the relative abundance of four OTUs and increased the relative abundance of three OTUs ($P < 0.05$; Figure 5). By contrast, litter addition significantly increased the relative abundance of two OTUs (Figure 5; Table S3). All of the significantly affected OTUs were in the family Glomeraceae apart from a single OTU in the Acaulosporaceae, which had lower relative abundance in the litter removal treatment. Neither litter treatment altered the total number of AM fungal OTUs (richness; ANOVA: $F_{2,12} = 0.15$, $P = 0.86$), nor the proportional abundance of the dominant AM fungal taxon (predominance; ANOVA: $F_{2,12} = 0.37$, $P = 0.69$; Figure S4). Of the variables fitted to the NMDS ordination, soil pH, effective base saturation (EBS), resin-extractable P, and inorganic N concentrations were significantly correlated with AM fungal community composition (Figure 4).

AM fungal community assembly

Litter manipulation moderately altered the degree of relatedness between taxa in AM fungal communities, as summarised by the Net Relatedness Index (NRI). There was a greater likelihood of detecting closely related taxa in litter addition plots than litter removal plots (ANOVA: $F_{2,12} = 4.02$, $P = 0.05$; Figure 6), although neither treatment differed significantly from controls. Furthermore, whereas neither treatment showed significant phylogenetic structure of AM fungal communities relative to null model distributions, the NRI was >0 in the litter addition treatment and <0 in the litter removal treatment, indicating a trend towards phylogenetic under-dispersion (taxa more related to each other than expected by chance) in the litter addition treatment (Figure 6) and phylogenetic over-dispersion (taxa less related to each other than expected by chance) in the litter removal treatment. However, when we used the Nearest Taxa Index (NTI), which is sensitive to patterns in relatedness close to the tips of the phylogeny, AM fungal communities were neither significantly structured relative to null distributions, nor affected by litter manipulation (ANOVA: $F_{2,12} = 0.25$, $P = 0.79$).

DISCUSSION

Litter removal altered AM fungal community composition (Figure 4), indicating that inputs of organic matter are important in structuring AM fungal communities. Together with substantially greater AM fungal root colonisation in the superficial organic layer than the mineral soil (70% versus 30% respectively; Figure 2e-h), our findings suggest that AM fungi obtain a substantial part of their nutrition from decomposing organic matter in this lowland tropical forest. We observed a trend towards increased AM fungal colonisation of roots growing in the mineral soil both in litter addition and litter removal treatments relative to controls (Figure 2), providing some support for our hypotheses that plants may increase investment in AM fungal associations in both litter addition and litter removal treatments.

Litter removal

Litter removal may have altered AM fungal community composition by reducing N-availability, either via direct fungal N-limitation, or by altering plant N status, leading to changes in plant allocation to AM fungi. Litter removal reduced the amount and availability of soil inorganic N, and crucially, reduced N concentrations in leaf litter after five years (Sayer & Tanner, 2010b; Sayer *et al.*, 2012), suggesting that N- availability to plants had decreased. N concentrations of AM fungal hyphae are substantially higher than that of plant tissues (Hodge *et al.*, 2010) and comparison of the C:N ratios of plant and fungal tissues indicate that severe N-limitation may be more likely to suppress fungal growth than plant growth (Kaye & Hart, 1997; Johnson, 2010). Given that different AM fungal taxa are known to vary in growth strategy and biomass allocation, and vary in the translocation of N to plant partners (Veresoglou *et al.*, 2012), litter removal may have selected for low-N AM fungal specialists. Alternatively, litter removal may have altered AM fungal community composition by increasing AM fungal competition (both with other AM fungi and saprobes) for a more limited resource.

An alternative possibility is that changes in AM fungal community composition in the litter removal plots reflect niche separation arising from a shift in AM fungal P-acquisition strategies. The availability of P is thought to limit many biological processes in lowland tropical forests (Vitousek & Sanford, 1986), and is a limiting nutrient in these forests (Wright *et al.*, 2011; Turner & Wright, 2013). As a large proportion of the P required for plant growth is cycled through leaf litter (Sayer & Tanner, 2010b), we would expect the litter removal treatment to affect plant P status. However, there was no reduction in leaf litter P in litter removal plots, nor a reduction in litterfall or plant productivity in the first 6 years of litter

manipulation (Sayer & Tanner, 2010b; Sayer *et al.*, 2012), indicating that trees in the litter removal plots were able to access sufficient P from alternative sources to maintain productivity and foliar P concentrations.

At least some of the additional P available to plants in the litter removal treatment was probably acquired from stable organic P pools in the mineral soil. Organic P in forests occurs in fresh organic matter (such as leaf litter), microbial biomass, and non-biomass stable organic phosphorus (Vincent *et al.*, 2010). Under normal conditions, P is rapidly released from leaf litter via leaching (Schreeg *et al.*, 2013) or mineralisation (Richardson & Simpson, 2011) before being taken up directly by plants by mycorrhizal fungi (Herrera *et al.*, 1978). This results in 'direct' nutrient cycling by which nutrient losses through leaching might be minimised (Went & Stark, 1968). After three years of litter removal, the stable organic P pool in the upper 2 cm of the mineral soil was reduced by 23%, while the overall inorganic P pool remained unchanged (Vincent *et al.*, 2010). Given that our study took place after nine years of litter removal, and the depletion of the stable organic P pool had conceivably continued, it is probable that additional P could also have been mobilised from recalcitrant mineral P stocks in the soil.

The role of AM fungi in P acquisition is well-known (Smith & Read, 2008), and is likely that a shift in plants' primary source of P from decomposing litter to stable organic P and stocks of mineral P would involve a change in the primary function of plants' AM fungal associations. Although limited, there is evidence that different AM fungal species differ in their ability to acquire (Cavagnaro *et al.*, 2005) and transport P to plant hosts (Munkvold *et al.*, 2004), and that AM fungal taxa may benefit plants to different degrees based on the type of soil P available (eg. mineral versus organic; Reynolds *et al.*, 2005). Consequently, it is possible that the taxa with increased relative abundance in litter removal plots were mineral P specialists, and those with decreased relative abundance were litter specialists (Figure 5). Nonetheless, it is striking that the dominant taxon and the relative abundances of most taxa in the litter removal treatment remained unchanged. Given the probable shift in plants' primary P source in the litter removal treatment, this would suggest that most of the AM fungal taxa observed at this site are readily able to adapt to the changed conditions. This is interesting in the light of studies of ectomycorrhizal fungi, which document wide differences in the ability of different taxa to mobilise and acquire P from different sources (Plassard *et al.*, 2011).

Other factors besides changes in nutrient availability could explain the shift in community composition observed in the litter removal treatment. Organic amendments such as leaf litter can affect a number of other soil properties besides nutrient availability, such as habitat space available for decomposers (Sayer, 2006). It is thus possible that AM fungal communities were affected by changes in the non-AM microbial community or soil fauna, which can impact AM fungal growth and function (Johnson *et al.*, 2005; Sayer *et al.*, 2006b; Gryndler *et al.*, 2008; Hodge, 2014), and which play a key role in AM fungal uptake of nutrients from leaf litter given the lack of documented saprophytic effects of AM fungi (Hodge, 2014). Previous studies at this site show no major changes in either temperature or soil water content among treatments (Sayer & Tanner, 2010a), and it is thus unlikely that these factors are responsible for the observed effects.

Soil pH was correlated with the NMDS ordinations of AM fungal community shifts, and may have been responsible for some of the observed shifts in community composition. However, studies documenting the effects of pH on AM fungi have largely reported a reduction in root colonisation and extra-radical hyphal biomass with decreasing pH (Wang *et al.,* 1993; Clark, 1997; van Aarle *et al.,* 2002) as well as reduced AM fungal β diversity (Dumbrell *et al.*, 2009), none of which were observed in this study.

Regardless of the mechanism underlying the shifts in AM fungal community composition, the trend towards more phylogenetically over-dispersed (less closely related) AM fungal communities in the litter removal plots relative to the litter addition plots (Figure 6) may reflect increasing competition between AM fungal taxa following litter removal. This is because more closely related AM fungal taxa tend to share functional traits (Maherali & Klironomos, 2007; Powell *et al.*, 2009), a phenomenon known as phylogenetic trait conservatism (Webb *et al.*, 2002). Consequently, phylogenetically over-dispersed communities are thought to be structured more by competition than by habitat filtering, which reduces the likelihood that closely related and functionally similar taxa will co-occur (Webb *et al.*, 2002).

Litter addition

AM fungal colonisation of roots was substantially higher in the organic horizons than the mineral soil in the control and litter addition treatments (70% versus 30% respectively; Figure 2e-h). This finding agrees with a sizeable body of evidence which shows that the

addition of organic material may increase AM fungal colonisation of plant roots (Gryndler *et al.*, 2005; 2008; Gosling *et al.*, 2010), and AM fungal sporulation (Gosling *et al.*, 2010). Indeed, AM fungal hyphae proliferate in organic substrates (Hodge & Fitter, 2010), and grow into decomposing leaf litter in tropical forests (Herrera *et al.*, 1978; Posada *et al.*, 2012; Camenzind & Rillig, 2013). Together with the finding that fine roots proliferated into the organic horizons in the litter addition treatment (Sayer *et al.*, 2006a), our results suggest that AM fungi may represent important pathways for plant uptake of nutrients from sites of organic matter decomposition in this tropical forest. However, given that AM fungi lack substantial saprophytic capability (Hodge, 2014), it is unlikely that AM fungi themselves are actively involved in litter decomposition, but rather are able to efficiently acquire nutrients as they are released from decomposing organic matter by the action of saprobes.

Given much greater root colonisation by AM fungi in the organic horizons of the litter addition and control plots relative to the mineral soil, it is surprising that we observed no significant increase in root colonisation in the mineral soil of litter addition treatments relative to controls (Figure 2a-d), where organic matter content is elevated relative to controls (Tanner *et al.*, 2016). It is possible that plant investment in AM fungi in litter addition plots is lower, due to the increases in soil fertility and tree nutrient status (indicated by marginal increases in litterfall and foliar N and P; Figure 1, Table 2; (Sayer & Tanner, 2010b; Sayer *et al.*, 2012). This interpretation follows from the functional equilibrium hypothesis, by which plants allocate resources to the structures that are the most helpful in acquiring the most limiting nutrients (Johnson, 2010), and by which plants should reduce investment in AM fungal associations when soil fertility increases because the carbon costs outweigh the nutritional benefits (Mosse & Phillips, 1971; Johnson, 2010). Reduced plant investment in AM fungi would counter the stimulatory effects of organic matter on AM fungal colonisation.

Limitations of this study

We did not measure NLFA in the superficial organic layer, or below 10 cm so we were not able to determine if total AM biomass was affected by litter treatment. In addition, we did not characterise AM fungal communities from roots sampled from the superficial organic layer due to technical constraints. As such, we are unable to address the extra-radical responsiveness of AM fungi to increased inputs of organic matter, and directly address the selection of litter-specific AM fungal communities. Vertical stratification of ectomycorrhizal

communities has been described in boreal forest podzols (Rosling *et al.*, 2003), and increased AM fungal colonisation of roots in the superficial organic layer could be hypothesised to reflect shifts in the structure and composition of AM fungal communities. This warrants further investigation. Finally, we made no direct measure of nutrient transfer, and our discussion of how leaf litter manipulation altered AM fungal function is thus necessarily speculative.

Potential sequencing bias

AM fungal communities were strongly dominated by taxa in the Glomeraceae (Figure 3), which was due in part to our choice of marker region because the SSU is biased towards Glomeraceae (Kohout *et al.*, 2014) and may underestimate diversity in some Diversisporales (Davison *et al.*, 2015). Indeed, a previous study in the Barro Colorado Nature Monument (BCNM) using Sanger sequencing and the same AM1/NS31 primer set similarly found a strong dominance of AM fungal species in the Glomeraceae (Husband *et al.*, 2002). Furthermore, a compilation of globally sampled AM fungal sequences obtained from the amplification of a similar SSU region (with the primers AML1/NS31) described a similar pattern: 79% of OTUs were from the order Glomerales (compared to 84% in this study), and 15% were from the Diversisporales (compared to 14% in this study; (Öpik *et al.*, 2013). By contrast, a study in a montane forest in Ecuador using the ribosomal large subunit (LSU) region found their dataset dominated by the Diversisporales (Camenzind *et al.*, 2014).

We used the number of DNA sequences as a measure of relative abundance of OTUs. Although sequence abundance may reflect biases introduced through PCR and sequencing protocols, the NS31-AM1 primer set exhibited very low levels of PCR bias when used to amplify artificial community templates of known composition (Cotton *et al.*, 2014). This is possibly because of the consistent length (c. 1.5% variation) and GC content (c. 3% variation) of the amplified region across different AM fungal taxa (Helgason *et al.*, 1999), as variation in amplicon length and GC content are known to cause biases in PCR reactions (Ihrmark *et al.*, 2012), and may cause biases in the 454 sequencing process as well (Kauserud *et al.*, 2011). In any case, in a comparative analysis of our dataset using both quantitative and presence-absence approaches led to identical conclusions (Figure S5).

CONCLUDING REMARKS

Our findings show that the presence of decomposing leaf litter is important both in structuring AM fungal communities, and in determining the extent of root colonisation by AM fungi. Alterations in AM fungal community composition in response to litter removal may be due to a range of factors including the reduction of key nutrients supplied by decomposing leaf litter, notably N and P, changes in the action of saprobes, and changes in water availability and pH. We hypothesise that a reduction in the quantity of decomposing fresh organic matter brought about by litter removal may lead to AM fungi obtaining scarce nutrients such as P from recalcitrant organic or mineral sources in the soil. Our hypothesis helps to explain how trees were able to maintain their P-status despite the chronic removal of a major P input in this lowland tropical forest, and merits further investigation.

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AUTHOR CONTRIBUTIONS

MS and SM designed the study. EVJT and EJS established the leaf litter manipulation experiment. MS and DR performed the lab and field work. PAO and HW supported the lipid analysis. BT conducted the nutrient analysis. NR conducted the bioinformatic analysis. MS conducted the statistical analysis and wrote the manuscript with input from all authors.

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Supporting Information

Figure S1. Effect of litter manipulation on the levels of NLFA 16:1ω5 in the top 10 cm of forest soil.

Figure S2. Rarefaction curves pooled by experimental treatment and for each sample.

Figure S3. Maximum-likelihood phylogenetic tree of all operational taxonomic units (OTUs) detected in this study.

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Figure S5. Comparison of AM fungal communities described by the quantitative Bray-Curtis metric of dissimilarity (a), the Jaccard presence-absence based metric of dissimilarity (b), and correlation between the two (c).

Table S1. Response of soil physical characteristics to nine years of litter removal and addition in a tropical forest.

Table S2. AM fungal OTUs altered by nine years of leaf litter addition and removal.

Table S3. Number of sequences per sample after blasting, filtering, merging and trimming.

936

937

938 **FIGURE LEGENDS**

939 Figure 1. Effects of litter manipulation on soil physical characteristics. Values are means ±
940 Fisher's Least Significant Difference. Grey shaded regions represent control treatments.
941 Litter treatments are significantly different from controls at $\alpha < 0.05$ ($n = 5$) where error bars
942 do not overlap the grey shaded regions. Standard normal deviates are plotted to facilitate
943 visual comparison of effect size. Al, Mn and N (inorganic) were log transformed prior to
944 analysis due to heteroscedasicity. N (inorg.) = inorganic N; P (res.) = resin extractable P; P
945 (tot.) = total P; TEB = total exchangeable bases; EBS = effective base saturation; L- = litter
946 removal treatment; L+ = litter addition treatment.

947

948 Figure 2. Percent root length colonised by AM fungi (total colonisation, colonisation by
949 hyphae, colonisation by vesicles and colonisation by arbuscules). Left-hand panels (a-d)
950 show the effect of litter manipulation on AM fungal colonisation of roots in the mineral soil.
951 Right-hand panels (e-h) compare colonisation in roots between the mineral soil ('soil') and
952 superficial organic horizon ('organic') across control and litter addition treatments. L- is litter
953 removal, C is control, and L+ is litter addition. In left hand panels (a-d) values are means ±
954 Fisher's Least Significant Difference, and non-overlapping error bars indicate significance at
955 $\alpha < 0.05$ ($n = 5$). In right-hand panels, values are means ± 95% confidence intervals obtained
956 by parametric bootstrapping with 10000 simulations.

957

958 Figure 3. Mean proportional abundance of AM fungal genera (a) and families (b) in mixed
959 root samples across litter manipulation treatments ($n = 5$); L- is litter removal, C is control,
960 and L+ is litter addition.

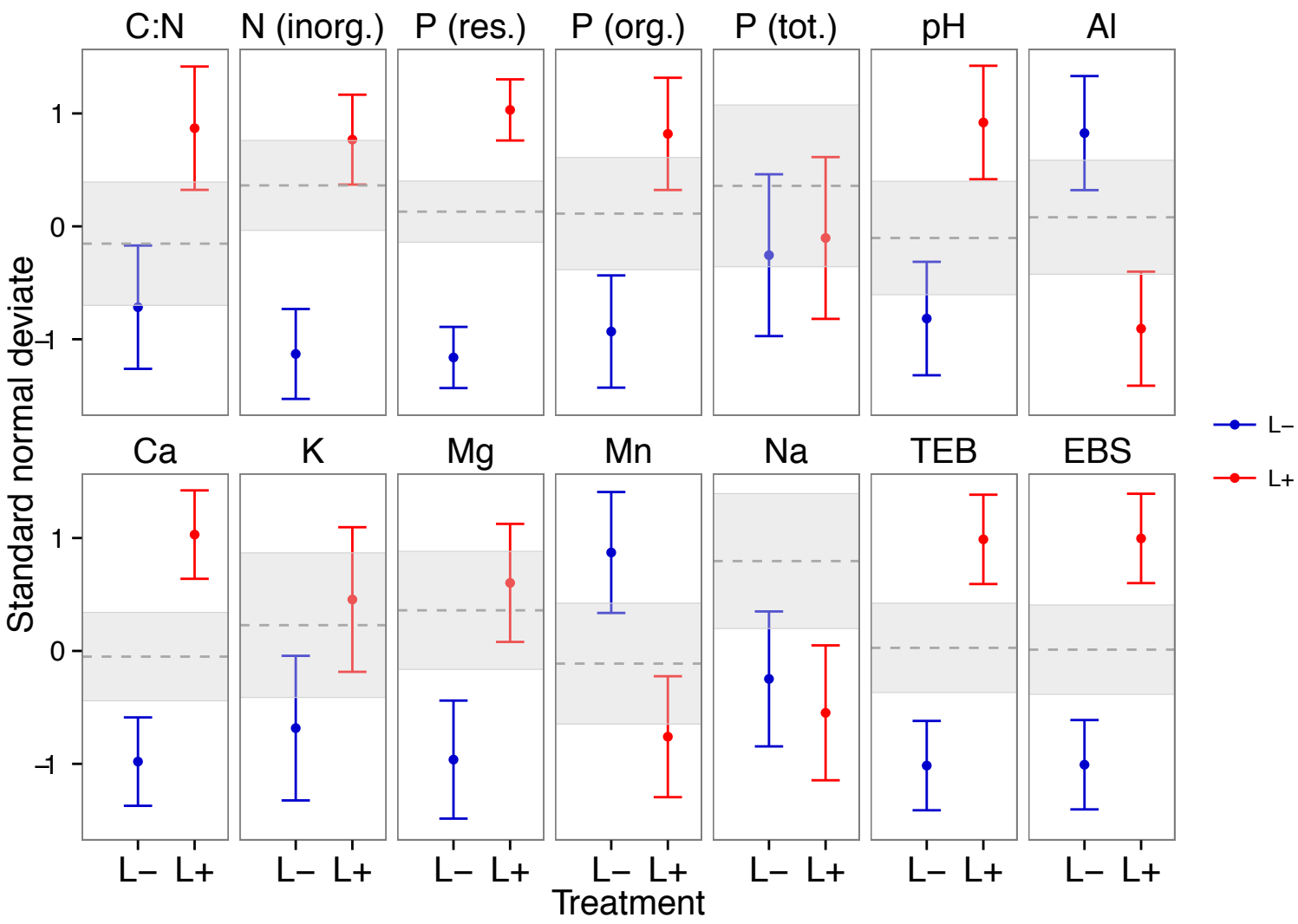
961

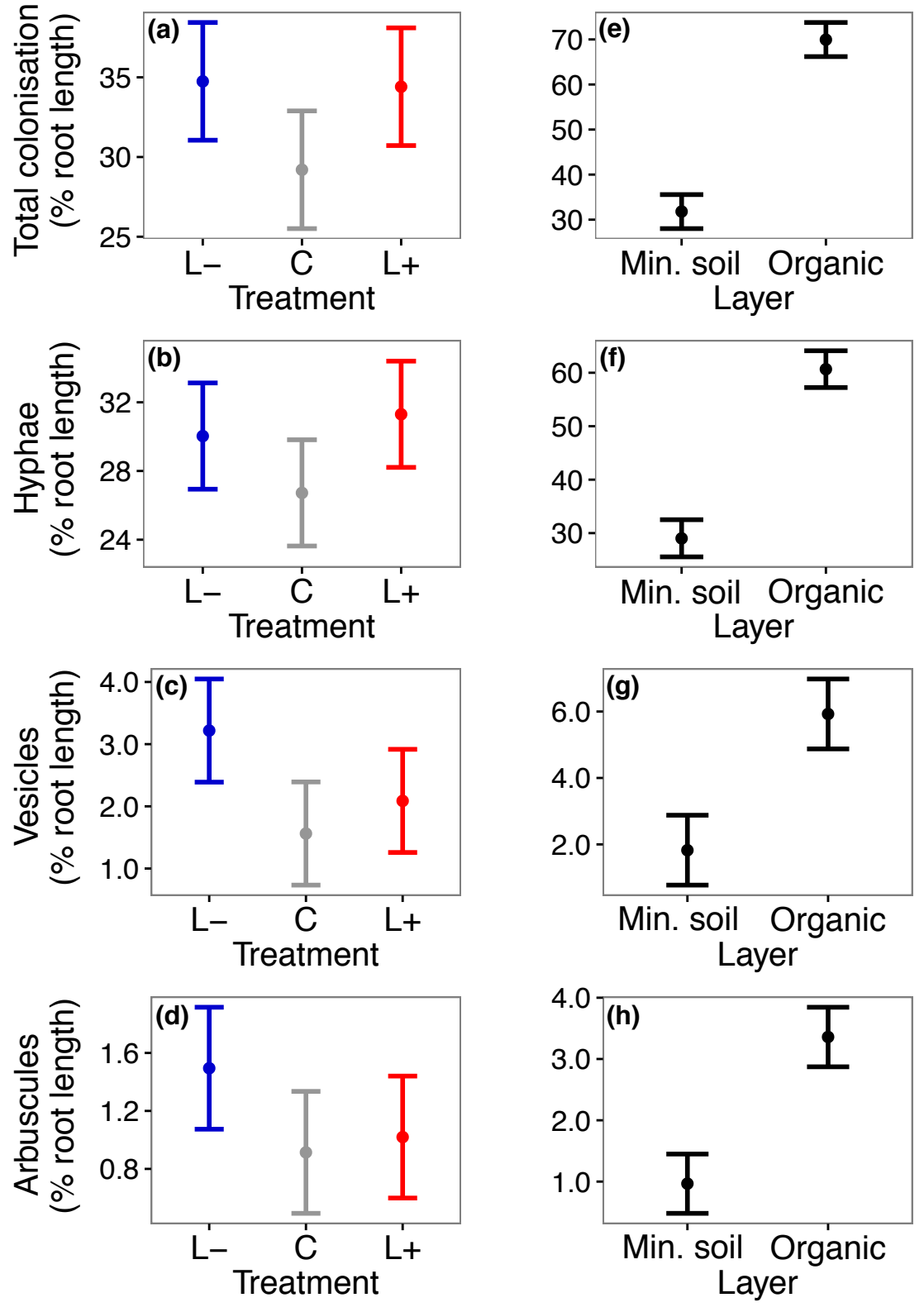
962 Figure 4. NMDS ordination plot showing changes in AM fungal community composition in
963 long-term litter removal plots (circles), but not litter addition plots (triangles), compared to
964 controls (squares) in a lowland tropical forest. Site scores are shown and ellipses describe

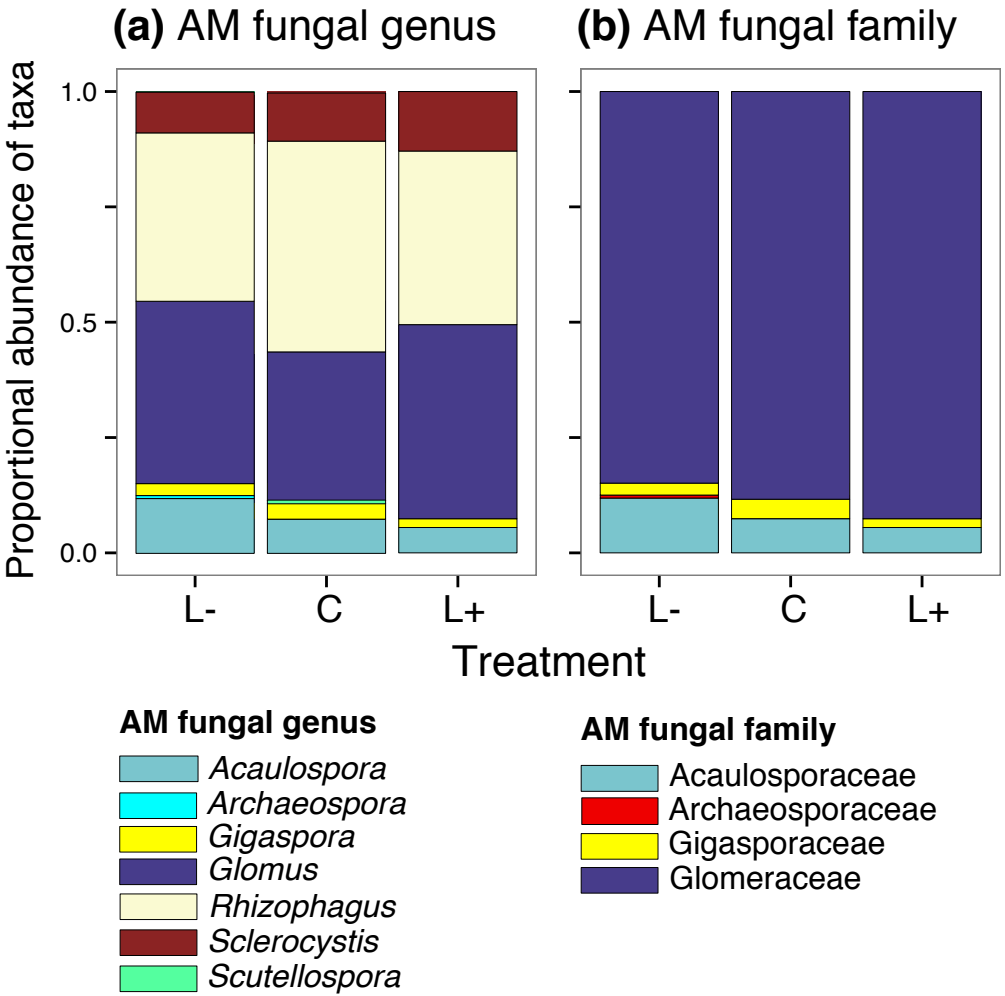
95% confidence areas. Arrows indicate the direction and degree of significant correlations between NMDS axes and soil physical characteristics (n = 5). EBS = effective base saturation; P (resin) = resin extractable phosphate; N (inorg.) = inorganic N; L- = litter removal, C = control, and L+ = litter addition. Axes are scaled to half-change (HC) units, by which one HC unit describes a halving of community similarity.

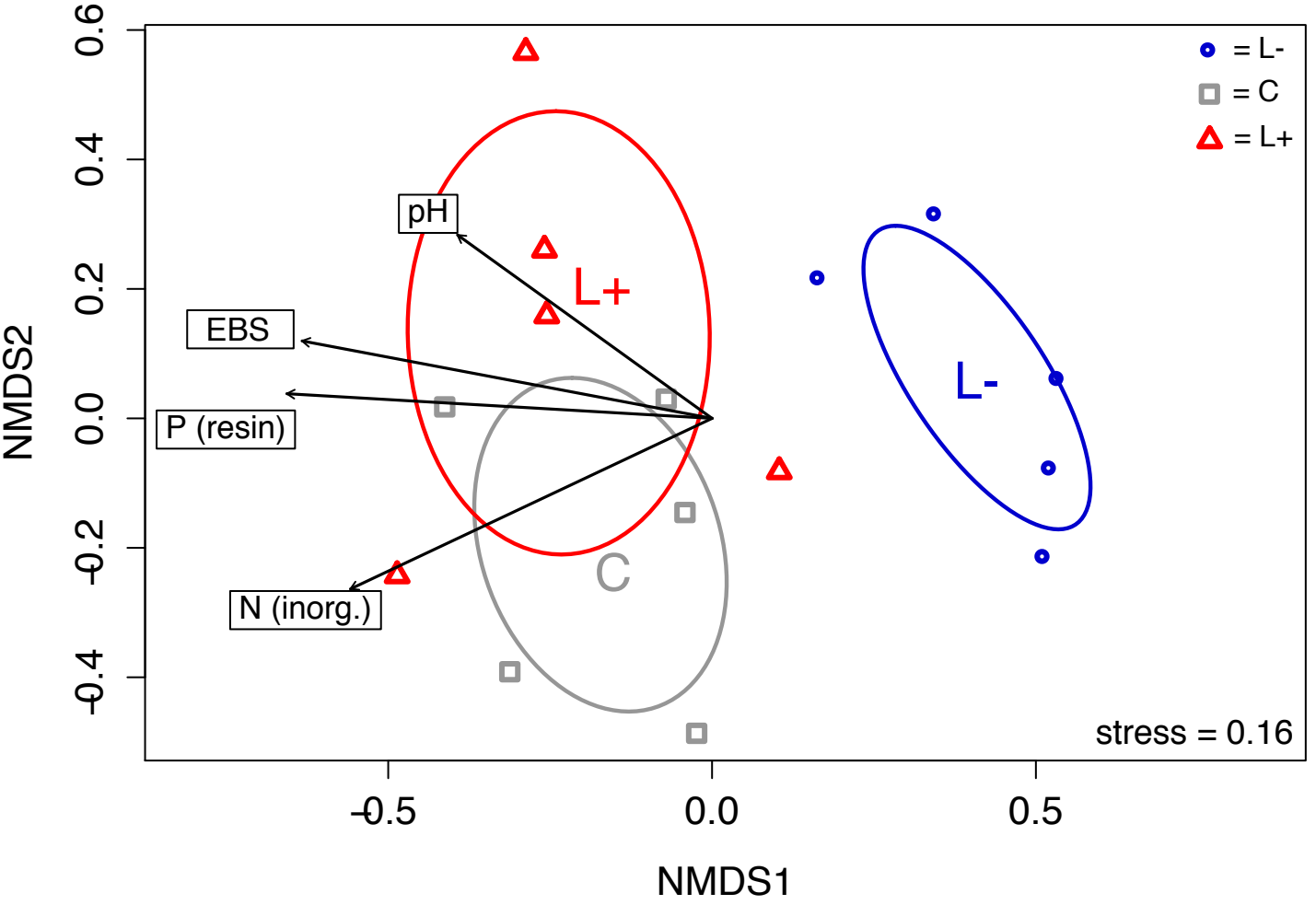
Figure 5. Effect of litter addition (red) and removal (blue) on the relative abundance of individual AM fungal operational taxonomic units (OTUs). Significantly altered ($P < 0.05$) OTUs are shown based on both adjusted and unadjusted P values. The names of OTUs that are significantly affected by litter manipulation are emboldened. x- axis indicates the effect size as log2 fold change, and error bars show standard errors. OTUs are arranged in order of decreasing rank abundance (more highly ranked OTUs are those that are more prevalent across all samples in the dataset). Significance was ascertained based on negative binomial Wald tests using standard maximum likelihood estimates for generalised linear models, as implemented in the DESeq2 package.

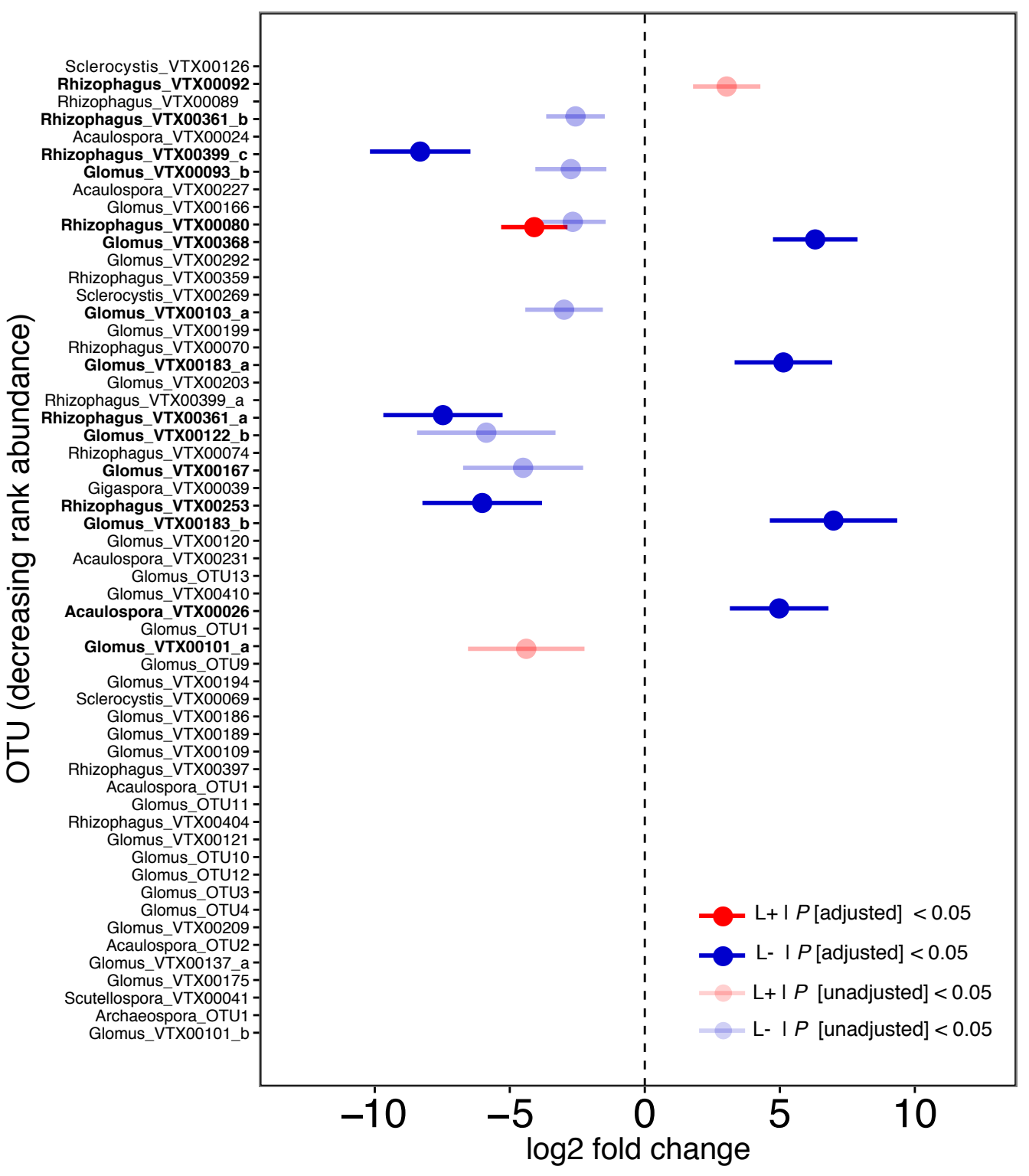
Figure 6. Litter manipulation moderately altered the degree of relatedness between taxa in AM fungal communities when described using the metric of Net Relatedness Index (NRI). Higher numeric values correspond to more closely related AM fungal communities. Values are means ± Fisher's Least Significant Difference: non-overlapping error bars indicate significance at $\alpha < 0.05$ (n = 5). Dotted lines indicate significance threshold of $\alpha = 0.05$ derived from comparison with 10000 null communities generated using the 'independentswap' algorithm. L- is litter removal, C is control, and L+ is litter addition.

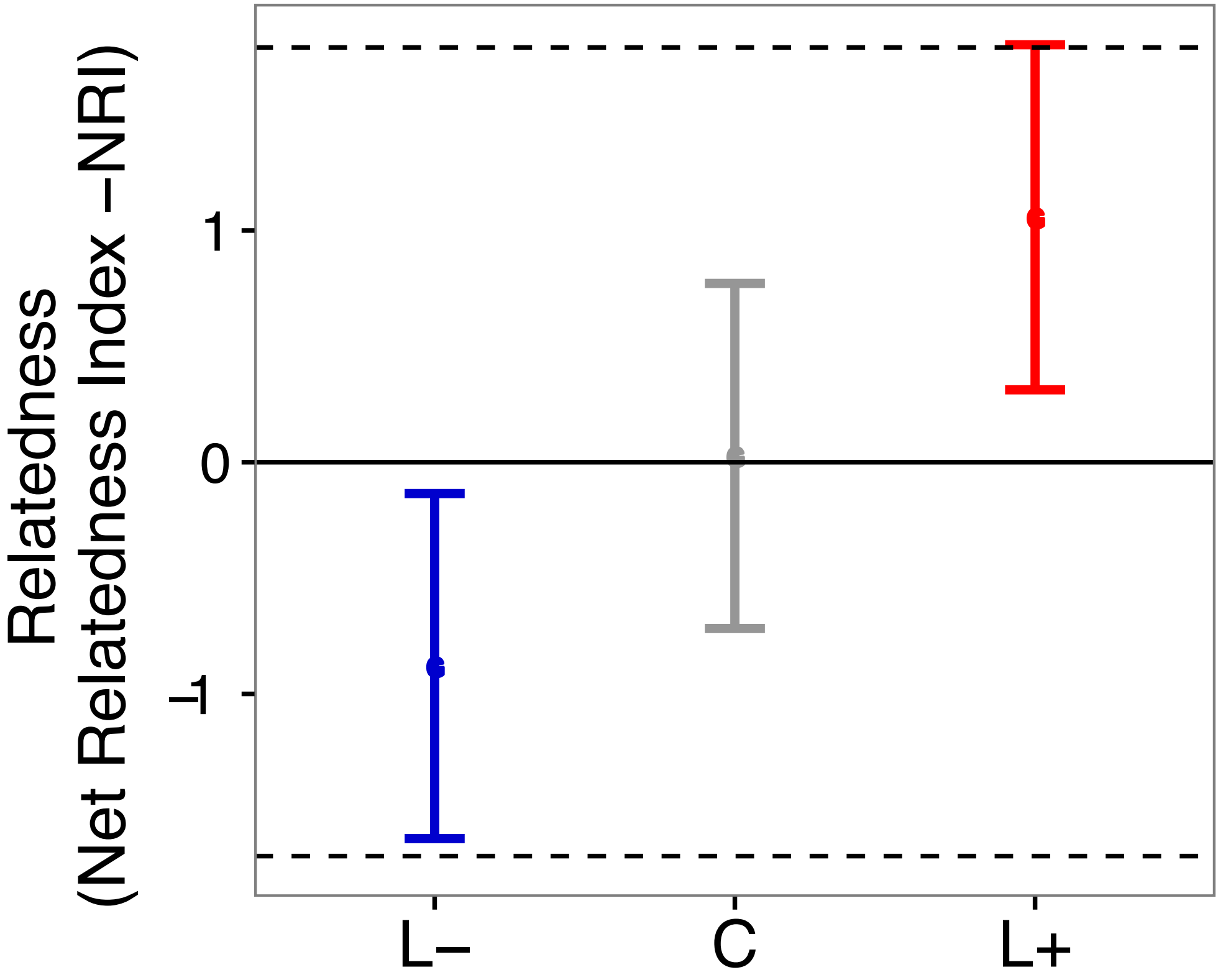

C:N
N (inorg.)
P (res.)
P (org.)
P (tot.)
pH
Al
Ca
K
Mg
Mn
Na
TEB
EBS
Standard normal deviate
1
0
−1
L−
L+
Treatment
L−
L+


(a)
Total colonisation (% root length)
35
30
25
L–
C
L+
Treatment
(e)
70
60
50
40
30
Min. soil
Organic
Layer
(b)
Hyphae (% root length)
32
28
24
L–
C
L+
Treatment
(f)
60
50
40
30
Min. soil
Organic
Layer
(c)
Vesicles (% root length)
4.0
3.0
2.0
1.0
L–
C
L+
Treatment
(g)
6.0
4.0
2.0
Min. soil
Organic
Layer
(d)
Arbuscules (% root length)
1.6
1.2
0.8
L–
C
L+
Treatment
(h)
4.0
3.0
2.0
1.0
Min. soil
Organic
Layer


(a) AM fungal genus
(b) AM fungal family
Proportional abundance of taxa
1.0
0.5
0.0
L-
C
L+
L-
C
L+
Treatment
AM fungal genus
Acaulospora
Archaeospora
Gigaspora
Glomus
Rhizophagus
Sclerocystis
Scutellospora
AM fungal family
Acaulosporaceae
Archaeosporaceae
Gigasporaceae
Glomeraceae


= L-
= C
= L+
pH
EBS
P (resin)
N (inorg.)
L+
L-
C
stress = 0.16
NMDS1
NMDS2
-0.5
0.0
0.5
-0.4
-0.2
0.0
0.2
0.4
0.6


OTU (decreasing rank abundance)
Sclerocystis_VTX00126
Rhizophagus_VTX00092
Rhizophagus_VTX00089
Rhizophagus_VTX00361_b
Acaulospora_VTX00024
Rhizophagus_VTX00399_c
Glomus_VTX00093_b
Acaulospora_VTX00227
Glomus_VTX00166
Rhizophagus_VTX00080
Glomus_VTX00368
Glomus_VTX00292
Rhizophagus_VTX00359
Sclerocystis_VTX00269
Glomus_VTX00103_a
Glomus_VTX00199
Rhizophagus_VTX00070
Glomus_VTX00183_a
Glomus_VTX00203
Rhizophagus_VTX00399_a
Rhizophagus_VTX00361_a
Glomus_VTX00122_b
Rhizophagus_VTX00074
Glomus_VTX00167
Gigaspora_VTX00039
Rhizophagus_VTX00253
Glomus_VTX00183_b
Glomus_VTX00120
Acaulospora_VTX00231
Glomus_OTU13
Glomus_VTX00410
Acaulospora_VTX00026
Glomus_OTU1
Glomus_VTX00101_a
Glomus_OTU9
Glomus_VTX00194
Sclerocystis_VTX00069
Glomus_VTX00186
Glomus_VTX00189
Glomus_VTX00109
Rhizophagus_VTX00397
Acaulospora_OTU1
Glomus_OTU11
Rhizophagus_VTX00404
Glomus_VTX00121
Glomus_OTU10
Glomus_OTU12
Glomus_OTU3
Glomus_OTU4
Glomus_VTX00209
Acaulospora_OTU2
Glomus_VTX00137_a
Glomus_VTX00175
Scutellospora_VTX00041
Archaeospora_OTU1
Glomus_VTX00101_b
L+ | P [adjusted] < 0.05
L- | P [adjusted] < 0.05
L+ | P [unadjusted] < 0.05
L- | P [unadjusted] < 0.05
−10
−5
0
5
10
log2 fold change


Relatedness
(Net Relatedness Index –NRI)
1
0
−1
L−
C
L+

Arbuscular mycorrhizal fungal community composition is altered by long-term litter removal but not litter addition in a lowland tropical forest

Supporting Information: Figures S1-S5 and Tables S1-S3

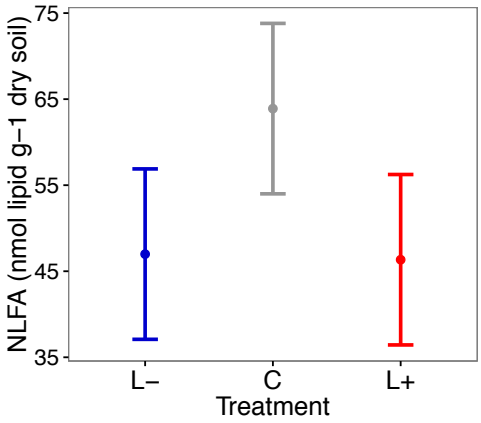


Figure S1. Effect of litter manipulation on the levels of NLFA 16:1ω5 in the top 10 cm of forest soil. Values are means ± Fisher's Least Significant Difference: non-overlapping error bars indicate significance at $P < 0.05$. L- is litter removal, C is control, and L+ is litter addition.

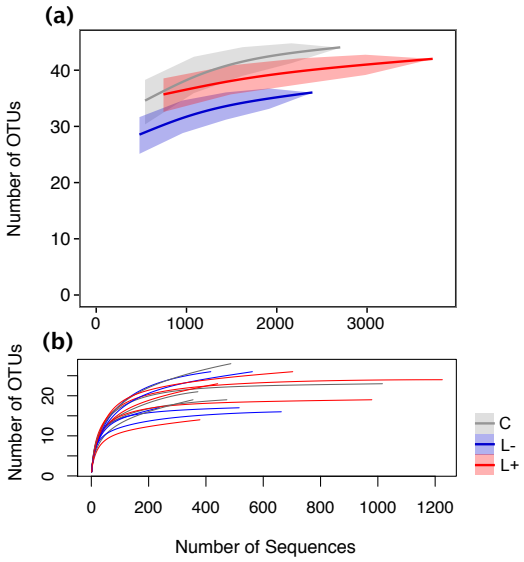


Figure S2. Rarefaction curves pooled by experimental treatment (a) approached asymptotes, indicating that sampling effort was sufficient to capture the range of AM fungal taxa across the sites. Rarefaction curves for each sample (b) indicated that sequencing intensity was sufficiently high to detect the majority of OTUs. C is control, L- is litter removal, and L+ is litter addition. Shaded bands show 95% confidence regions calculated from the standard error of the estimate using the function specaccum in the R package vegan.

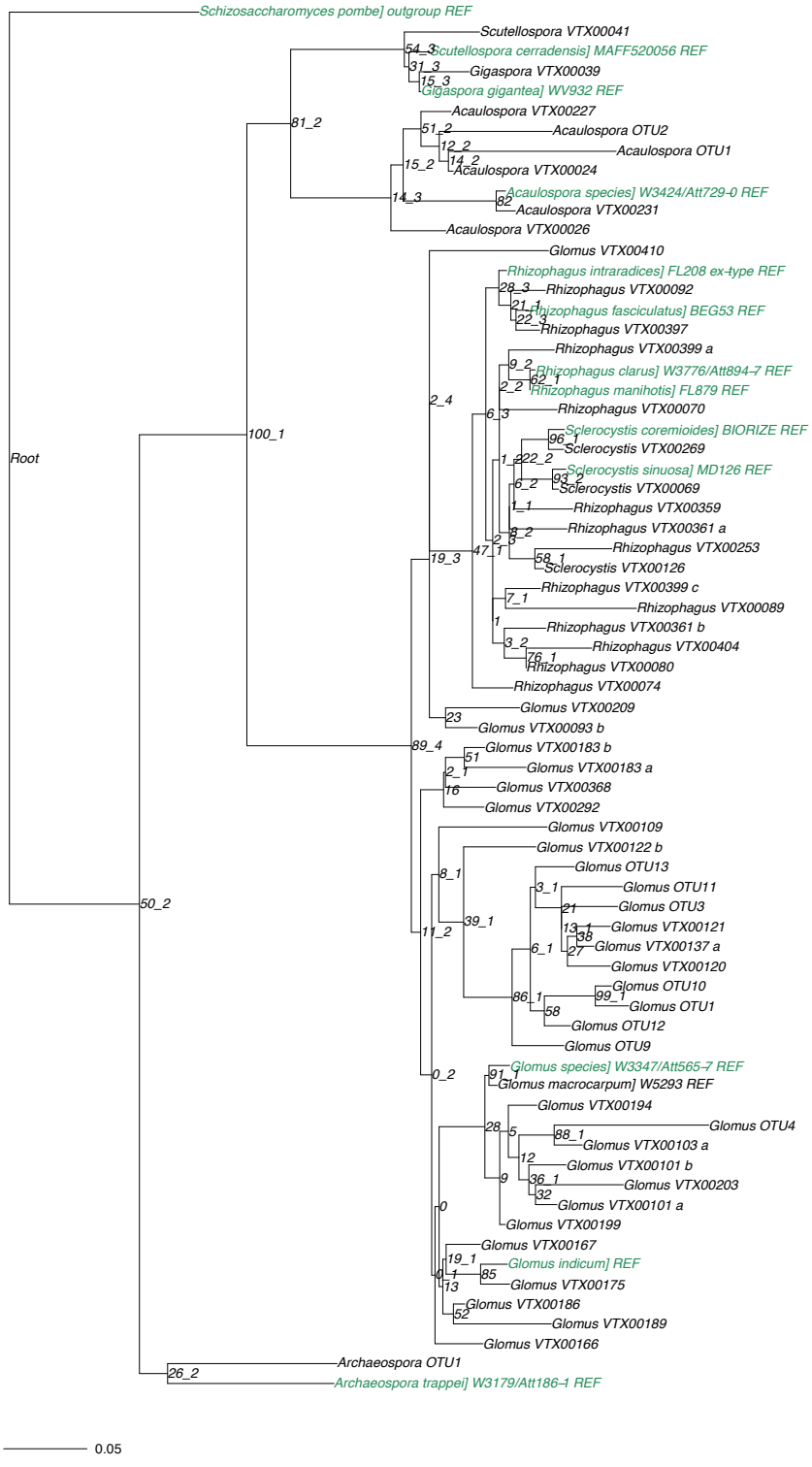


Figure S3. Maximum-likelihood phylogenetic tree of all operational taxonomic units (OTUs) detected in this study. The scale bar equals the number of substitutions per site. A subset of reference sequences from Kruger *et al.* (2012) are displayed in green text.

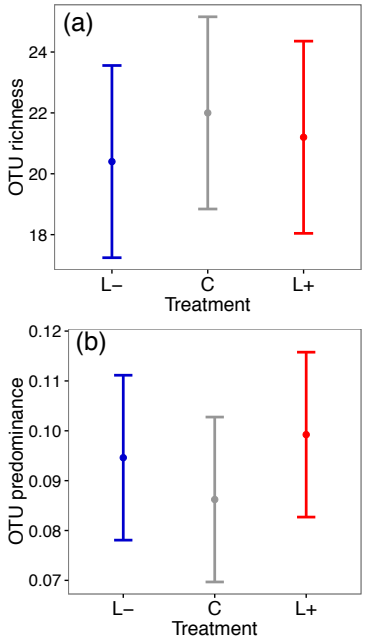


Figure S4. Effect of litter manipulation on AM fungal OTU richness (total number of OTUs in a sample; a) and predominance (the proportional abundance of the dominant AM fungal taxon; b). Values are means ± Fisher's Least Significant Difference: non-overlapping error bars indicate significance at $P < 0.05$. L- is litter removal, C is control, and L+ is litter addition.

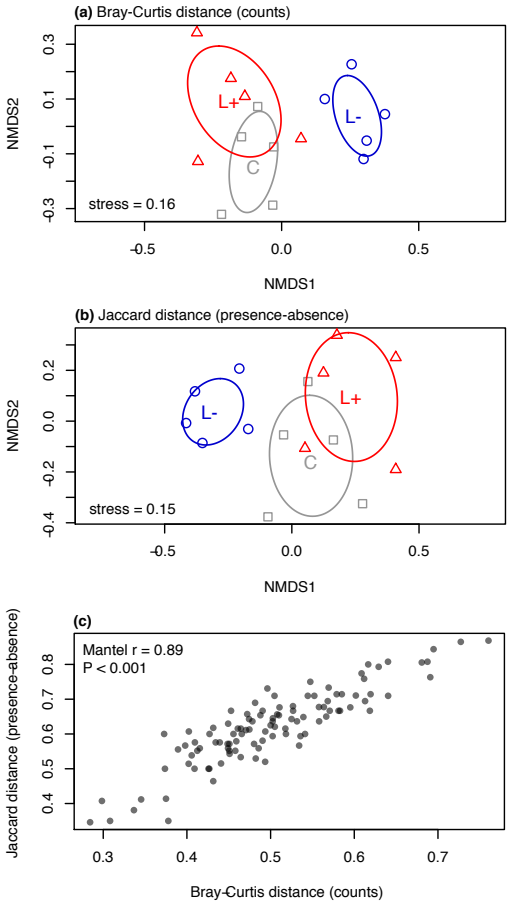


Figure S5. Comparison of AM fungal communities described by the quantitative Bray-Curtis metric of dissimilarity (a), the Jaccard presence-absence based metric of dissimilarity (b), and correlation between the two (c). (a) and (b) are two-dimensional NMDS plots with ellipses describing 95% confidence areas around the sample scores.

Table S1. Response of soil physical characteristics to nine years of litter removal and addition in a tropical forest

Variable	Treatment (means: *n* = 5)					SE
	Litter removal		Control	Litter addition		
Al	38.0		12.0	2.6		1.7
Ca	740	*	1400	2200	*	180
K	53.0		75.0	80.0		9.7
Mg	270	*	390	410		31
Mn	72.0		46.0	34.0		1.2
Na	9.8		13	8.7	*	1.4
P (resin)	6.0	*	17.0	25.0	*	1.5
P (total)	370		390	370		20
P (organic)	76		93	100		5
C:N	9.9		10.0	11.0		0.2
N (inorganic)	2.1	*	4.9	6.1		1.2
TEB [1]	6.1	*	10.0	14.0	*	1.1
EBS [2]	7.0	*	11.0	15.0	*	1.0
pH	5.3		5.5	5.8		0.1

Notes

Variables significantly affected by litter addition are asterisked

All nutrients are expressed as mg kg^{-1} of dry soil

TEB is expressed as cmol kg^{-1} dry soil

EBS is a unitless fraction

SE = standard error

[1] Total Exchangeable Bases

[2] Effective Base Saturation

Table S2. AM fungal OTUs altered by nine years of leaf litter addition and removal, as ascertained using the DESeq2 package.

AM fungal OTU	log2 Fold Change	SE [1]	*P* value		Rank abundance	Treatment [2]	Direction of change
Acaulospora_VTX00026	-4.98	1.83	0.03	*	32	L-	-
Glomus_VTX00183_a	-5.13	1.81	0.03	*	18	L-	-
Glomus_VTX00183_b	-6.99	2.36	0.02	*	27	L-	-
Glomus_VTX00368	-6.31	1.57	0.00	*	11	L-	-
Rhizophagus_VTX00253	6.02	2.22	0.03	*	26	L-	+
Rhizophagus_VTX00361_a	7.47	2.21	0.01	*	21	L-	+
Rhizophagus_VTX00399_c	8.32	1.86	0.00	*	6	L-	+
Rhizophagus_VTX00080	4.09	1.23	0.04	*	10	L+	+
Glomus_VTX00093_b	2.74	1.32	0.04		7	L-	+
Glomus_VTX00103_a	2.99	1.44	0.04		15	L-	+
Glomus_VTX00122_b	5.87	2.56	0.02		22	L-	+
Glomus_VTX00167	4.51	2.22	0.04		24	L-	+
Rhizophagus_VTX00080	2.67	1.22	0.03		10	L-	+
Rhizophagus_VTX00361_b	2.57	1.09	0.02		4	L-	+
Glomus_VTX00101_a	4.39	2.16	0.04		34	L+	+
Rhizophagus_VTX00092	-3.03	1.25	0.02		2	L+	-

Asterisks (*) denotes *P* values corrected for multiple comparisons

[1] SE = standard error

[2] L- is litter removal and L + is litter addition

Table S3. Number of sequences per sample after blasting, filtering, merging, and trimming (exclusion of OTUs with a total of 5 or less reads)

Plot	Treatment	Number of sequences
1	C	1016
11	C	372
15	C	473
5	C	355
7	C	487
12	L-	516
13	L-	417
4	L-	237
6	L-	663
8	L-	562
10	L+	441
14	L+	379
2	L+	979
3	L+	1225
9	L+	703
		total = 8825

References

Krüger M, Krüger C, Walker C, Stockinger H, Schüßler A. **2012**. Phylogenetic reference data for systematics and phylotaxonomy of arbuscular mycorrhizal fungi from phylum to species level. *New Phytologist* **193**: 970–984.