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Nutrient enrichment increased species richness of leaf litter fungal assemblages in a tropical forest

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Abstract

Microbial communities play a major role in terrestrial ecosystem functioning, but the determinates of their diversity and functional interactions are not well known. In this study, we explored leaf litter fungal diversity in a diverse Panama lowland tropical forest in which a replicated factorial N, P, K and micronutrient fertilization experiment of 40 × 40 m plots had been ongoing for nine years. We extracted DNA from leaf litter samples and used fungal-specific amplification and a 454 pyrosequencing approach to sequence two loci, the nuclear ribosomal internal transcribed spacer (ITS) region and the nuclear ribosomal large subunit (LSU) D1 region. Using a 95% sequence similarity threshold for ITS1 spacer recovered a total of 2523 OTUs, and the number of unique ITS1 OTUs per 0.5–1.0 g leaf litter sample ranged from 55 to 177. *Ascomycota* were the dominant phylum among the leaf litter fungi (71% of the OTUs), followed by *Basidiomycota* (26% of the OTUs). In contrast to our expectations based on temperate ecosystems, long-term addition of nutrients increased, rather than decreased, species richness relative to controls. Effect of individual nutrients was more subtle and seen primarily as changes in community compositions especially at lower taxonomic levels, rather than as significant changes in species richness. For example, plots receiving P tended to show a greater similarity in community composition compared to the other nutrient treatments, the +PK, +NK and +NPK plots appeared to be more dominated by the *Nectriaceae* than other treatments, and indicator species for particular nutrient combinations were identified.

Keywords: diversity, fungi, leaf litter, LSU, macronutrients, micronutrients, rDNA ITS

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Introduction

In tropical terrestrial ecosystems, more than 80% of net aboveground primary production returns to the forest floor as litter (Swift *et al.* 1979). This litterfall provides substrate for leaf litter fungal assemblages that include ecological guilds such as saprotrophs, endophytes, parasitic and pathogenic fungi and a few mycorrhizal fungi. Fungi, along with bacteria and invertebrate animals, are important consumers in the brown (or detrital) food webs (Kaspari *et al.* 2008). While litter decomposition is mediated by both biotic and abiotic processes, leaf litter fungal decomposers, in particular, play an important biotic role in recycling ecosystem nutrients (Schneider *et al.* 2012).

Many recent studies and reviews have investigated the effect of macronutrients, primarily nitrogen (N), on fungal communities. These studies have demonstrated a general decrease in fungal species diversity and shifts

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in microbial communities as a result of nutrient enrichment (Avis *et al.* 2003, 2008; Treseder 2004, 2008; Parrent *et al.* 2006; Cox *et al.* 2010). However, these studies primarily focused on ectomycorrhizal fungi in temperate and boreal ecosystems. In addition, most nutrient addition studies only span a few years. Compared with soil and ectomycorrhizal fungal diversity studies, leaf litter fungal diversity and community studies have not been as extensive (Peay *et al.* 2008; Edwards & Zak 2010; Edwards *et al.* 2011). However, fungal decomposers have been studied on leaf litter in the tropics (Lodge 1997; McGuire *et al.* 2012) and in temperate regions (Blackwood *et al.* 2007; Lindahl *et al.* 2007; U'Ren *et al.* 2010; Voříšková & Baldrian 2012).

Two loci were amplified in this study, the nuclear ribosomal internal transcribed spacer (ITS) region, the formal fungal barcode (Schoch *et al.* 2012), and the nuclear ribosomal large subunit (LSU) D1 region. The ITS region approximately delimits species, allowing for a finer-scale taxonomic resolution (Horton & Bruns 2001), while the more conserved LSU gene region is appropriate for phylogenetic inference and taxonomic questions above the genus level (Kõljalg *et al.* 2005; Porter & Golding 2012). We choose the combination of these two gene regions because together they provide a near species-level resolution (ITS) and a higher-level phylogenetic insight into the leaf litter community (LSU).

Here, we investigate the taxonomic richness and diversity of leaf litter fungal assemblages in a diverse tropical lowland forest in Panama in which nutrient additions have been manipulated for nine years. We used a factorial N, P and K fertilization of 40 × 40 m plots, along with four additional plots with a micronutrient treatment (i.e. B, Ca, Cu, Fe, Mg, Mn, Mo, S and Zn). The goals of this study were to determine the effect of increased nutrients on leaf litter fungal community structure, and with the factorial design, we expected to determine which nutrients or nutrient combinations were the strongest drivers of fungal community structure in these tropic leaf litter habitats. Based on previous fungal nutrient addition studies, we hypothesized that nutrient addition would decrease fungal species richness. However, it should be noted this hypothesis is based mainly on N addition studies, and our sites are low in K and P. Therefore, we did also anticipate to see a response of fungal assemblages to +K and +P plots.

Materials and methods

Study site

Leaf litter samples were collected from the Smithsonian Tropical Research Institute's Gigante Fertilization Experiment (9°06′31″ N, 79°50′37″ W) in the Barro Colorado Nature Monument (BCNM) on the Gigante peninsula, Republic of Panama. Thirty-six 40 × 40 m plots were established in 1998 along a macronutrient (NPK) factorial experiment on the Gigante peninsula within the macronutrient fertilization plot consisting of eight treatments and four replicates: nitrogen (N), phosphorus (P), potassium (K), NP, NK, PK, NPK, micronutrients (M) and control plots (n = 36). These plots were compared with control plots that did not receive fertilization, to evaluate possible nutrient effects on leaf litter fungal assemblages in a diverse lowland tropical forest. The factorial NPK fertilization (i.e. N, P, K, NP, NK, PK, NPK and control) with four replicates was laid out across 32 plots in a stratified random design (Fig. S1, Supporting information). The factorial design also allowed us to look for interactions among macronutrients. The plots have received nutrient additions since 1998 and were sampled in 2007. The plots are approximately 30–40 m apart and span over 27 ha. The forest is categorized as a lowland primary rainforest (older than 300 years; Leigh 1999). The soils are characterized as oxisol soils low in available P and K (Yavitt & Wieder 1988; Cavelier 1992). Fertilizers were applied four times a year during the wet season (beginning in June 1998) to reach the following total doses for each year's four applications: 125 kg N per ha/year (as coated urea [$(NH_2)_2CO$]), 50 kg P per ha/year (as triple superphosphate [$Ca(H_2PO_4)_2.H_2O$)]) and 50 kg K per ha/year (as KCI). Similar doses are used in forestry (Binkley 1986) and have also been used in tropical montane studies [100–150 kg N, 50–65 kg P, 50 kg K (e.g. Tanner *et al.* 1992; Vitousek *et al.* 1995)]. An additional four '+M' plots were dosed with a micronutrient fertilizer (Scott's S.T.E.M) consisting of HBO_2, $CuSO_4$, $FeSO_4$, $MnSO_4$, $ZnSO_4$ and $(NH_4)_6Mo_7O_{24}$ at 25 kg per ha/year plus dolomitic limestone $CaMg(CO_3)_2$ (36.8 kg/year) at 230 kg per ha/year (Kaspari *et al.* 2008).

Sampling

Four replicate samples were collected in September 2007 from the eight treatments [nitrogen (N), phosphorus (P), potassium (K), NP, NK, PK, NPK, micronutrients (M)] and control plots (n = 36). Sampling from each plot consisted of a 10 × 10 cm quadrant of leaf litter located in the centre of the plot. Leaf litter was collected down to mineral soil and then sifted for 30 s through a 1-cm screen in an effort to exclude soil and nonleaf litter matter. Samples were processed within 12 h or stored at 3 °C for 24 h and then processed.

DNA extraction

Total DNA was isolated from 0.5 to 1.0 g (wet wt.) from each homogenized and ground litter sample using the

MOBIO Power Soil DNA Extraction Kit (MOBIO Laboratories, Carlsbad, CA, USA) according to manufacturer's protocols. DNA was extracted from two replicate subsamples, pooled for each sample and then stored at −20 °C until needed.

454 pyrosequencing methods

PCR amplification. Both the ITS region (ITS1F/ITS4) (White *et al.* 1990; Gardes & Bruns 1993) and the LSU region [LROR_F (Amend *et al.* 2010)/LR5-F (Tedersoo *et al.* 2008)] were PCR amplified ($n = 36$) with 8-bp barcodes. The forward primers included the 'A' pyrosequencing adaptor along with the 8-bp multiplex tag and forward gene-specific primer (5′-A + 8 bp multiplex tag + forward gene-specific primer-3′), and the reverse primers included the 'B' pyrosequencing adaptor along with the reverse gene-specific primer (5′-B + reverse gene-specific primer-3′) for the ITS and LSU loci regions, respectively (Amend *et al.* 2010). Each PCR consisted of 1.2 units of HotStarTaq polymerase (Qiagen), 2.5 μL of PCR buffer (containing 50 mM KCL; 10 mM Tris; 2.5 mM $MgCl_2$; and 0.1 mg/mL gelatin), 0.2 mM dNTPs, 0.5 μM of each primer, 2.5 μL of DNA and H_20 to a final concentration of 25 μL. Thermocycling in an Eppendorf Mastercycler Gradient thermocycler was carried out under the following conditions: initial denature for 10 min at 95 °C, 34 cycles of 1 min at 95 °C, 1 min at 51 °C (ITS) or 54 °C (LSU) and 1 min at 72 °C and final extension for 7 min at 72 °C. PCR products were purified with the Qiagen QIAquick 96 PCR Purification Kit (Qiagen, Valencia, CA, USA) following the manufacturer's instructions. Purified PCR products were quantified with the Invitrogen Qubit Fluorometer (Invitrogen, Carlsbad, CA, USA) and pooled in equimolar concentrations by locus and then sequenced on separate ¼ plate (ITS) and ¼ plate (LSU) of a 454 Titanium sequencing run (454 Life Sciences/Roche Applied Biosystems, NJ, USA; Margulies *et al.* 2005). Samples were sequenced at the Duke Institute for Genome Sciences and Policy.

Sequence processing and analyses

Sequencing half of a plate with two loci—(1/4 of a plate) ITS and (1/4 of a plate) LSU—resulted in 157 364 ITS sequences and 31 023 LSU sequences total. Reads were quality controlled using the QIIME software (Caporaso *et al.* 2010), with a minimum length of 200 bp and maximum length of 650 bp for both ITS and LSU and were filtered with the following default parameters in QIIME: 0 number of allowable Ns, maximum homopolymer length of 6 and an average Q score of at least 25. All QIIME commands were executed using MacQIIME version 1.2.1-20110224a. The ITS1 region was verified and extracted using the ITS1 extractor for fungal ITS sequences (Nilsson *et al.* 2010), and the D1 region was verified and extracted using a novel LSU-tailored version of V-Xtractor (Hartmann *et al.* 2010; Fig. S2, Supporting information; http://www.microbiome.ch/web/Tools.html). Within the program QIIME, operational taxonomic units (OTUs), a representation of species (ITS) or higher taxonomic groups (LSU), were determined at 90–99% sequence similarity using UCLUST (Edgar 2010) and plotted against number of OTUs defined by each cut-off level to determine where sequence error level became problematic. Singletons (OTUs represented by a single sequence) are often artefactual in pyrosequencing data and were therefore removed from subsequent analyses for both the ITS1 and LSU D1 data sets (cf. Tedersoo *et al.* 2010). Because singletons were removed, Chao1 and similar species estimators that are sensitive to singletons were not used for species estimation. Raw sequences were deposited in the Sequence Read Archives (Kodama *et al.* 2012) of NCBI under accession number SRA059446 for ITS and SRA060238 for LSU and were queried against the fungal ITS rDNA sequence database and the fungal large subunit (LSU) database curated by the Fungal Metagenomic Project (http://www.borealfungi.uaf.edu/) (Lee Taylor, James Long, Shawn Houston). Both databases are compiled from GenBank, AFTOL and TreeBASE. The BLAST (version 2.2.18) output was imported into MEGAN 4.3 (Huson *et al.* 2007) using default parameters. Nonfungal taxa and BLAST hits that resulted in no matches were removed from the analysis. Only samples with ≥ 950 sequences per treatment for the ITS1 region and samples with ≥ 250 LSU D1 sequences were included in analyses in an effort to include all samples.

Diversity and statistical analyses

Alpha and beta diversity analyses were conducted on the confirmed fungal taxa in QIIME. Total study rarefaction and individual sample rarefactions were also calculated using EstimateS 8.0 (Colwell 2009). Alpha diversity indices, including Shannon diversity (H′) (Magurran 1988), Simpson (Simpson 1949), phylogenetic diversity (PD) (Faith 1992; Faith & Baker 2006) and Fisher's alpha (Fisher *et al.* 1943) for each sample, were computed in QIIME. A Mantel test (Mantel 1967; Legendre & Fortin 1989) was computed to test for spatial autocorrelation. The Mantel test (using both the Jaccard index and unweighted UniFrac), ANOVA, ANOSIM and an indicator species analysis using the multipatt algorithm in the Indicspecies package (De Cáceres & Legendre 2009) were computed in the R programming environment (R Core Development Team, RDCT 2005).

In addition to the univariate analysis, we compared the effects of N, P and K to exploit the factorial structure of the experiment (SAS 2006). Nonmetric multidimensional scaling (NMDS) ordinations (using the Jaccard index) were used to represent the dissimilarities in community composition among samples using the Vegan package (Dixon 2003; Oksanen *et al.* 2010) in R. To test for a unimodal function, we summed OTU richness across fertilization treatments (control, one-nutrient, two-nutrient and three-nutrient treatments), and we applied a Kruskal–Wallis rank sum test ($P < 0.05$) in R.

The LSU D1 extracted sequences were aligned using the PyNAST (a python implementation of the NAST alignment algorithm) in QIIME using LSU sequences from James *et al.* (2006) as the core alignment template. FastTree (Price *et al*. 2009) was used as the tree building method in QIIME. Community phylogenetic dissimilarity (using LSU D1) was calculated using the unweighted UniFrac metric (significant at Bonferroni corrected, $\alpha \leq 0.05$) in QIIME. All analyses were based on the extracted sequences data sets (i.e. ITS1 or LSU D1) unless otherwise clearly stated.

Results

454 pyrosequencing results

Among the ITS sequences, 40 033 sequences were removed due to reads that were too short or that contained ambiguous bases, low mean quality score or long homopolymers. This left a total of 117 331 ITS sequences (median length 523 bases, range 200–650 bp) that passed the default QIIME quality control steps. Among the LSU sequences 7293 were removed due to reads that were too short or contained ambiguous bases, low mean quality score or long homopolymers. This left a total of 23 730 LSU sequences (median length 533 bases, range 200–630 bp) that passed the default QIIME quality control steps.

The entire ITS amplicon, the entire LSU amplicon and the extracted ITS1 spacer and extracted D1 regions were assigned to operational taxonomic units (OTUs) based on 90.0–99.0% sequence similarity and plotted against the number of OTUs that were determined. This approach illustrated that unextracted sequences yield higher numbers of OTUs than the extracted variable region (i.e. ITS1 and LSU D1 regions) at all similarity thresholds. In addition, the number of OTUs for all regions is initially linear but increases as bin size for OTU similarity is narrowed. The sharp exponential increase in OTU numbers seen at the narrowest bin sizes is likely a result of sequence error or possibly intraspecific variation. For this reason, OTUs were conservatively determined at 95% sequence similarity based on the accumulation of OTUs at different sequence similarities (Jumpponen & Jones 2009; Kunin *et al.* 2009; Jumpponen *et al.* 2010a,b) (see note in discussion) (Fig. S3, Supporting information). This threshold yielded a total of 3299 OTUs. After nonfungal taxa and sequences with no BLAST hits were removed, 2523 OTUs remained from the extracted ITS1 sequences. This threshold also yielded a total of 724 fungal OTUs for the LSU D1 extracted gene region. Results presented here are based on the extracted sequences data sets (i.e. ITS1 and LSU D1).

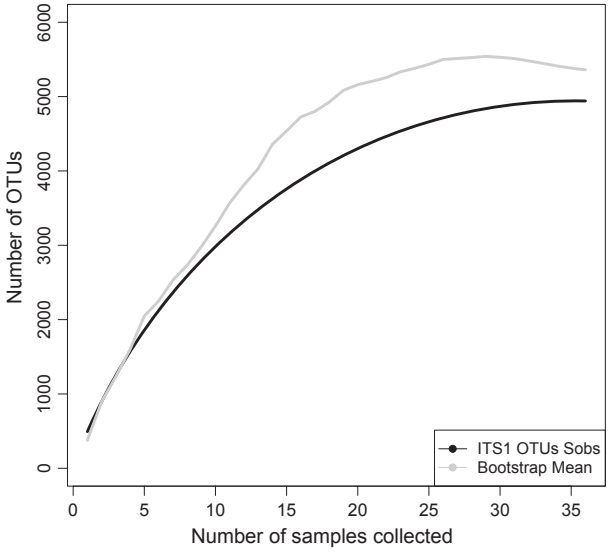

Fig. 1 Species accumulation curve of total study area. ITS1 OTUs clustered conservatively at 95% sequence similarity.

Diversity results

An accumulation curve of all the ITS1 OTUs in the study did level off, indicating the leaf litter community was sufficiently censused (Fig. 1). Bootstrap estimates for the rarefaction curves for each sample of the control, M, N, P and K treatments approach an asymptote at around 1500 sequences indicating that our sequencing effort captured a large proportion of the leaf litter diversity among the different treatments. However, all samples were rarified to 950 sequences to include all the samples and the rarefaction curves have not begun to level off yet (Fig. 2).

Alpha diversity indices of the total study area (ITS1) by treatment are presented in Table 1. There was a significant difference observed among Fisher's alpha [ANOVA: $F_{8,26} = 3.18$ ($P < 0.05$)]. However, no significant difference was observed among observed OTUs or for the bootstrap estimates (based on a one-way ANOVA) among all nutrient treatments or among only the control, M, K, P and N treatments. Based on a three-way ANOVA of the

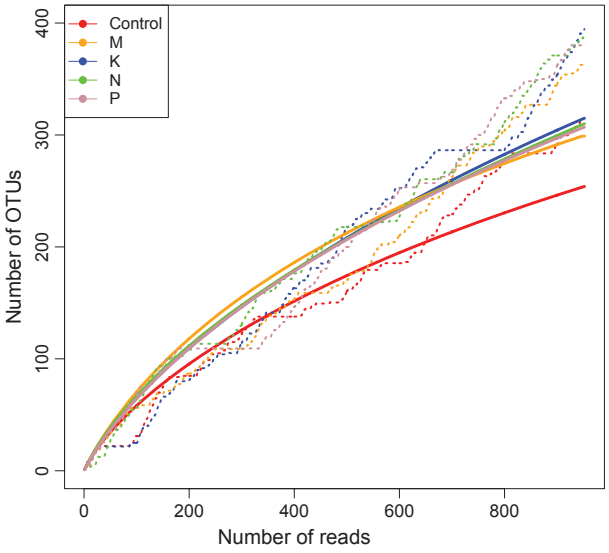

Fig. 2 Rarefaction curves of observed ITS1 OTUs per treatment. The control plots—C (red) plots have fewer OTUs compared with the plots that received fertilization treatments. Potassium—K (blue), nitrogen—N (green) and phosphorus—P (purple) plots have similar number of OTUs, while micronutrients—M (orange) plots have fewer OTUs. The dotted lines represent the bootstrap estimate.

bootstrap estimates with N, P and K as factors, there is an N*K interaction ($P < 0.05$) and a marginal P*K interaction (Table 1). The LSMeans describing the interaction show that adding N or K increases diversity above control levels, but adding both reduces diversity. We see the same interaction with P (Table 1). There were some nonsignificant trends in the patterns of diversity. Control plots had the lowest Shannon diversity compared to treatments that received fertilization. The control plots also had the fewest number of observed OTUs, followed by the +NPK plots. PK fertilization plots had the highest Shannon and Simpson diversity indices, along with the total highest number of observed OTUs, followed by +K, +N and +P plots. The control plots also had the lowest number of observed OTUs and PD in the LSU data set.

Taxonomic diversity analysis

Among the ITS1 OTUs, the *Ascomycota* (including approximately 29 orders, 44 families and 88 genera) were the dominant phylum (71% of the OTUs), followed by *Basidiomycota* (26% of the OTUs) (including approximately 31 orders, 38 families and 52 genera), *Glomeromycota* (2% of the OTUs), *Chytridiomycota* (1% of the OTUs), *Neocallimastigomycota* (<1%), *Blastocladiomycota* (<1%) and subphyla *Mucoromycotina* (<1%) [following (Hibbett *et al*. 2007) (Fig. 3)]. Two genera of the former *Zygomycota*, *Basidiobolus* (5 OTUs) and *Olpidium* (1 OTU), were also represented. Fifty-three per cent of OTUs were present in only one sample and only 1.3% of OTUs were present in half or more of the samples, indicating fairly even composition of these assemblages with no real dominants. Of the 1.3% of ITS1 OTUs present in half or more of the samples, all but one were ascomycetes, and the single exception was a *Mycena* species (Basidiomycota). LSU OTUs exhibited essentially the same pattern at the phylum level: *Ascomycota* (72%), *Basidiomycota* (24%), *Glomeromycota* (<1%) and *Chytridiomycota* (3.6%) (data not shown).

At the class level, the majority of ITS1 OTUs were *Sordariomycetes* (30% of the OTUs), followed by *Agaricomycetes* (24% of the OTUs), *Dothideomycetes* (14% of the OTUs), *Eurotiomycetes* (9% of the OTUs) and *Lecanoromycetes* (3% of the OTUs) (Fig. 3). The LSU gene region again had similar distribution of sequences across the class taxonomic level: *Sordariomycetes* (23%), *Agaricomycetes* (22%), *Dothideomycetes* (19%), *Eurotiomycetes* (4%) and *Lecanoromycetes* (3%). Basidiomycete saprotrophic families, such as *Agaricaceae*, *Marasmiaceae*, *Tricholomataceae*, *Tremellaceae* and *Corticiaceae s.l.*, were well represented in this study in both the ITS1 and LSU data sets.

The distribution of higher taxonomic levels was very similar across the treatments (control, M, N, P, K, NP, NK, PK and NPK), and only those represented by 3% of the OTUs or less were apparently variably present. For example at the phylum level, *Chytridiomycota* (1% of the overall sample) were not represented in the +PK, +NP, +NK or +NPK treatments. At the class level, there was a similar distribution of ITS1 OTUs between the treatments, although the +PK, +NK and +NPK have fewer representatives across different classes (e.g. no *Orbiliomycetes*, *Tremellomycetes* or *Glomeromycetes*). At the order level, no *Xylariales*, *Polyporales*, *Auriculariales* and *Tremellales*, and at the family level, no *Marasmiaceae* and *Agaricaceae* were detected on the +PK, +NP and +NPK plots. There is a slight increase in *Sordariomycetes* and a decrease in *Agaricomycetes* in these plots compared to the control plots. *Chytridiomycetes* are only present in +M, +P and +K. *Saccharomycetes* are only found in +N and +NP plots. There is a shift in microbial communities across different nutrient treatments, with the +PK, +NK and +NPK plots appearing to be more dominated by the *Nectriaceae* and the *Cortinariaceae (s.l.)* than other treatments at lower taxonomic levels (Fig. S4, Supporting information).

Treatment analysis

The number of ITS1 OTUs per rarified sample (presence/absence) ranged from 55 to 177. We analysed patterns of species composition in two ways. First, a

Table 1 Alpha diversity indices of treatments [ITS1 OTUs] based on rarified samples. (A) One-way ANOVA: Fisher's alpha was significant ($P < 0.05$). No significant difference was observed among observed species or bootstrap estimate based on a one-way ANOVA. (B) Three-way ANOVA: based on bootstrap estimates, there is a significant N*K ($P < 0.05$) interaction and a marginal P*K interaction

A

Treatment	Shannon	Simpson	OTUs sobs	Boostrap OTUs estimates	Fisher's alpha
Control	5.13	0.90	254	313	45.00
M	5.96	0.95	299	363	69.17
P	5.96	0.91	307	381	93.74
K	5.75	0.88	315	395	101.49
N	6.23	0.96	310	387	97.24
NK	6.06	0.96	298	369	79.54
PK	6.40	0.97	330	409	88.85
NP	6.22	0.95	287	355	99.39
NPK	5.55	0.91	285	349	75.11

B

Dependent variable: bootstrap

Source	d.f.	Sum of squares	Mean square	*F* value	*Pr* > *F*
Model	7	32688.93	4669.85	1.70	0.1557
Error	24	65788.26	2741.18		
Corrected total	31	98477.19			

Source	d.f.	Type III SS	Mean square	*F* value	*Pr* > *F*
N	1	414.22	414.22	0.15	0.7009
P	1	318.21	318.21	0.12	0.7363
N*P	1	6954.63	6954.63	2.54	0.1243
K	1	131.83	131.83	0.05	0.8283
N*K	1	12468.18	12468.18	4.55	0.0434*
P*K	1	11547.06	11547.06	4.21	0.0512
N*P*K	1	854.81	854.81	0.31	0.5817

N K	Least square mean	Standard error	*Pr* > |*t*|
0 0	214.39	18.51	<0.0001
0 1	249.81	18.51	<0.0001
1 0	261.06	18.51	<0.0001
1 1	217.52	18.51	<0.0001

P K	Least square mean	Standard error	*Pr* > |*t*|
0 0	215.58	18.51	<0.0001
0 1	249.51	18.51	<0.0001
1 0	259.87	18.51	<0.0001
1 1	217.82	18.51	<0.0001

comparison of the single-factor treatments (+N, +P, +K, M and control) with NMDS shows the +P treatments to be tightly clustered, +N and +K to be loosely clustered and micronutrient and the controls to be highly dispersed (Fig. S5, Supporting information). In the NMDS plot of all the treatments combined, the +P sites also clump closely together (Fig. S6, Supporting information). There is a general similarity (or clumping) of plots that have received macronutrient fertilization (primarily the +K, +P, +NP, +PK and +NPK sites) in the NMDS plot. Indicator species analysis (Table 2) points to 12 taxa with a high affiliation to 6 different fertilization treatments.

Distribution of fungal communities in nonmetric dimensional space (Fig. 4), analysed in a factorial ANOVA, points to compositional changes driven by the presence of N, P and K. Both N and P communities differ from plots receiving neither N or neither P, along NMDS1 (Fig. 4 and Table S1, Supporting information). Moreover, these diverge in different directions from the

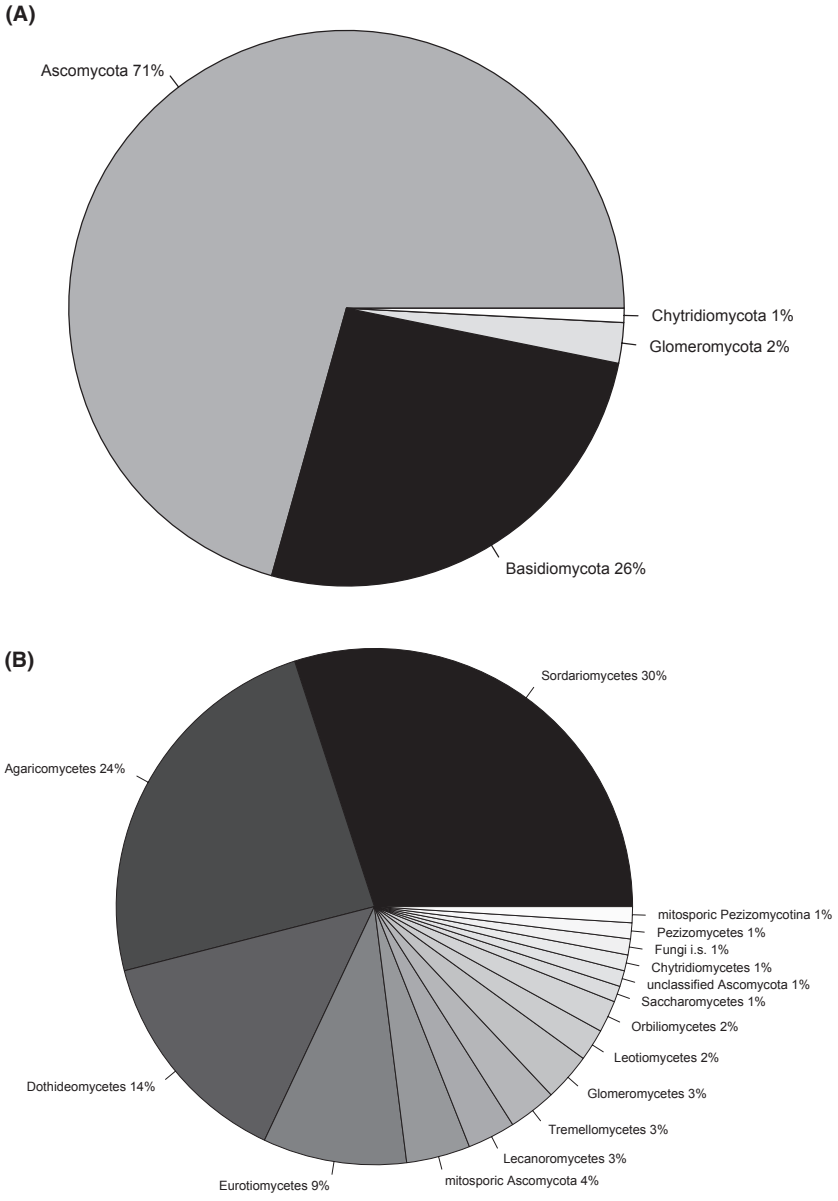

Fig. 3 (A) Distribution of total leaf litter fungal ITS1 sequences at the phylum taxonomic level. Ascomycota represent the majority of ITS1 OTUs. (B) Distribution of total leaf litter fungal ITS1 sequences at the class taxonomic level.

centroid suggesting different taxa accumulate on N vs. P plots. In NMDS4, N, P and K plots all three communities diverge suggesting a uniform effect of fertilization (Fig. 4 and Table S1, Supporting information).

A Mantel test was run to test for correlations between the species data distance matrix (LSU D1) and the raw geographic distances of the sites. Neither the Jaccard nor the unweighted UniFrac measures of community similarity were significant, indicating species composition is not related to geographic distances (Jaccard: Statistic = −0.0664, $P = 0.799$; unweighted UniFrac: statistic = −0.0034, $P = 0.504$). Analysis of similarities (ANOSIM) of the unweighted UniFrac metric (for LSU D1) was not significant ($R = 0.07$, $P = 0.153$), indicating that nutrient fertilization does not significantly partition phylogenetic community variance. Twenty-one per cent of all treatment pairwise UniFrac beta diversity significant tests were significant (including: control-N; control-P; K-N; K-NK; M-N; N-P). This test indicated that +N treatment plots are phylogenetically statistically significant from the control, +K, +P and +M treatment plots.

Discussion

This study revealed high species diversity of leaf litter fungi in the tropics. Interestingly, recent soil studies have demonstrated a dominance of *Basidiomycota* taxa (Buée *et al.* 2009; Jumpponen *et al.* 2010a). Here, we report a dominance of *Ascomycota*, followed by *Basidiomycota* among leaf litter fungi (Fig. 3). A dominance of

Table 2 Description of statistically significant indicator species

	Nutrient Plots						
	OTU number	Statistic	*P*-value	Taxonomic group	Best BLAST match	Match ID	% ID
K plots	1555	0.875	0.022	Xylariales	Hypoxylon	FM209467.1	99
	536	0.866	0.004	Hypocreales	Stephanonectria	EU273554.1	98
	1248	0.707	0.039	Hypocreales	Stachybotrys	AF08143.2	97
M plots	2338	0.816	0.007	Lecanorales	Lecanorineae	FJ554208.1	73
N plots	3191	0.764	0.011	Sordariales	Chaetomium	JF439468.1	94
	3440	0.750	0.024	Ascomycota	Uncultured ascomycota	AY273312.1	93
	3809	0.742	0.018	Rhizophydiales	Rhizophydium	JN943817.1	90
NK plots	2570	0.791	0.013	Auriculariales	Hyaloriaceae	HQ021773.1	79
NP plots	4491	0.879	0.001	Sordariales	Unclutured Chaetomiaceae	EU372832.1	84
	1914	0.866	0.005	Agaricales	Pluteus	FJ774083.1	86
	2111	0.740	0.049	Xylariales	Anthostomella	JQ411351.1	100
PK plots	3101	0.826	0.009	Chaetosphaeriales	Thozetella	AY330996.1	91

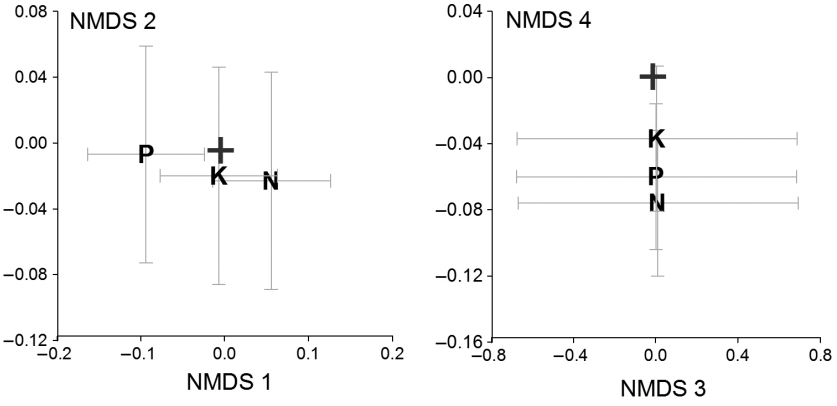

Fig. 4 Distribution of fungal communities in nonmetric dimensional space (LSMeans ± 2 standard errors). Cross represents the centroid.

Ascomycota in leaf litter has also recently been reported for both tropical and temperate studies (McGuire *et al.* 2012; Schneider *et al.* 2012; Voříšková & Baldrian 2012). High-throughput 454 pyrosequencing has allowed for greater sampling depth and has provided a greater insight into fungal richness and diversity across the study site, compared with a previous T-RFLP study (Kaspari *et al.* 2010).

The addition of nutrients impacted the richness and diversity of the leaf litter fungal assemblages on the plots receiving fertilization compared with the control plots. Control plots for both the ITS1 and LSU D1 data sets had the lowest observed species (OTUs) and bootstrap estimated richness, along with the lowest phylogenetic diversity (PD) for the LSU data set compared to the other plots that received nutrients. The interaction of nutrients on the +NPK plots resulted in low diversity (as defined by alpha diversity indices—Shannon and PD). There was no significant difference among observed species or bootstrap estimate (ITS1 OTUs) across the different nutrient treatments. However, there was a significant difference for Fisher's alpha ($P < 0.05$) based on a one-way ANOVA across the different nutrient treatments. There was also a significant N*K interaction ($P < 0.05$) and a marginal P*K interaction (Table 1) based on a three-way ANOVA.

Contrary to our hypothesis, there was an increase in fungal richness in more nutrient-rich plots (i.e. plots receiving fertilization). However, two- and three-fertilizer treatments (+NK, +PK and +NPK) had lower richness compared with single fertilizer treatments. (The one exception is the +PK plots, which had greater richness). We summed OTU richness across fertilization treatments (control, one-nutrient, two-nutrient and three-nutrient treatments) and applied a Kruskal–Wallis rank sum test, which indicated a unimodal function. This unimodal or 'humped-shaped' relationship between resource availability (i.e. nutrient fertilization) and diversity follows the productivity–diversity hypothesis (Tilman 1982; Tilman *et al.* 1996), which proposes that species richness is a unimodal function of productivity and that the availability of resources limits species diversity. In other words, diversity increases with resource availability up to a point and then decreases

as resources continue to increase. Waldrop *et al.* (2006) were the first to show this common relationship among biotic communities with soil fungi.

The addition of potassium, nitrogen and phosphorous all increased species richness. It is particularly interesting that nitrogen increases species diversity of leaf litter fungi because many studies have demonstrated a decrease in fungal species diversity with nitrogen addition (Avis *et al.* 2003, 2008; Treseder 2004, 2008; Parrent *et al.* 2006; Cox *et al.* 2010). However, as mentioned previously, most of these studies have focused on ectomycorrhizal communities in boreal and temperate regions. Blackwood *et al.* (2007) also found nitrogen addition did not have an effect on fungal litter communities in a temperate ecosystem. It is interesting to note that the natural soils in the Gigante plots are P and K limited (Walker & Syers 1976; Yavitt & Wieder 1988), whereas N is hypothesized to be abundant in lowland tropical forests (Santiago *et al.* 2012 and citations within). Treatment plots receiving P tended to show a greater similarity in community composition compared to the other nutrient treatments (Figs S5 and S6, Supporting information).

The addition of nutrients had a stronger effect on the taxonomic composition of the leaf litter fungal communities at lower taxonomic levels (i.e. family, genus and species), compared to higher taxonomic levels (i.e. phylum, class and order) (Fig. S4, Supporting information). However, there is an increase in ascomycetes on the plots receiving fertilization compared to the control plots, with an exception of the +NP plots. There is an increase in *Hypocreales* (and most notably within the family *Nectriaceae*) on +PK, +NK and +NPK plots (Fig. S4, Supporting information). Two *Hypocreales* species were significantly associated with +K plots, according to the indicator species analysis (Table 2). Interestingly, it appears that when K is combined with N or P, there is a decrease in diversity, and this may be due to an increase in *Hypocreales* species on these plots (Fig. S4, Supporting information, Table 2). Within the order *Hypocreales*, there are known to be plant pathogens and endophytes as well as saprotrophs. In fact, within the tropics, *Hypocreales* have been reported as dominate endophytes (Arnold & Lutzoni 2007). In addition, there is also an increase in *Cortinariaceae* (*s.l.*) (only represented by 6 OTUs) on +PK, +NK and +NPK plots (Fig. S4, Supporting information). Upon a closer look, three of the OTU sequences blasted to the genus *Cortinarius*, an ectomycorrhizal genus common in boreal forests (Taylor *et al.* 2010). However, OTU identifications below the family level should be interpreted with caution, at least until databases are populated with more sequences from the tropics.

Overall, both the ITS and LSU gene regions produced similar results. The use of two separate gene regions for the analysis provides a complimentary look at the data. OTUs were determined conservatively at 95% sequence similarity as a function of sequence similarity and in an effort to avoid overestimating richness, which is inherent with 454 pyrosequencing technologies compared with traditional sequencing methods (Huse *et al.* 2007; Kunin *et al.* 2009; Quince *et al.* 2009). The extracted ITS1 region is more variable, compared to the entire ITS region, which is another reason for choosing a more conserved sequence similarity. The ITS data set was re-analysed with both the ITS and ITS1 regions clustered at 97%, which is typically used for OTU delimitation of the entire ITS region (O'Brien *et al.* 2005; Amend *et al.* 2010; Tedersoo *et al.* 2010). Similar patterns resulted (data not shown) in the community analysis, and the major difference was the number of OTUs. In addition, the ITS and LSU data sets were queried against different databases for comparison, and again, similar patterns resulted.

We know from earlier studies in these same plots that nutrient additions increase decomposition rates. Specifically, Kaspari *et al.* (2008) saw cellulose decomposition enhanced 49% on +P plots and 30% on +K plots, and leaf litter decomposition was 30% faster on +P plots. They also found element concentrations increased up to 27% of +P plots, 34% on +K plots and 7% on +N plots, all without an increase in litterfall. Interestingly, there have been no discernible changes in plant composition on these plots after years of fertilization. However, there has been an increase in herbivory on leaves in +P and +K plots (Santiago *et al.* 2012) and a significant decrease in fine-root biomass with the addition of K (Wright *et al.* 2011). Whether the relatively subtle changes in fungal composition that we observed were responsible for these enhanced decomposition rates is not clear. It seems equally plausible that nutrient additions would accelerate decomposition even if fungal assemblages remained constant, but only further manipulative experiments would resolve this. In any case, the replicated factorial N, P, K, NP, NK, PK and NPK fertilization experiment combined with high-throughput pyrosequencing provided us with a refined understanding of how leaf litter fungal assemblages in a lowland tropical forest respond to the long-term addition of macro- and micronutrients and how the increase in these nutrients affects diversity and community composition. However, the relatively subtle effects of the nutrient additions on composition of these communities mean that there are likely to be other important factors that are driving the general patterns of leaf litter fungal diversity. Distance alone does not appear to be an important factor at this scale as there was not significant correlation found in the Mantel test. Earlier cultural studies suggest that a strong correlation might be expected with host leaves on litter fungal diversity

(Lodge 1997), but high-throughput culturing from two host species found little consistency with host (Polishook *et al.* 1996). McGuire *et al.* (2012) found no correlation between tropical leaf litter fungal communities and plant diversity or precipitation; however, they did see an increase in soil fungal diversity with increasing precipitation. Thus, much still remains to be explained about the patterns of high diversity in tropical fungal litter communities.

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M.K. and B.S. designed the research and collected samples; J.K. performed research and wrote the core parts of the article; H.N. and M.H. developed the modified version of V-Xtractor software; A.A. and T.B. helped with molecular techniques and analyses; and J.K. and M.K. analysed data. All co-authors assisted in the writing of the article.

Data accessibility

DNA sequences: NCBI SRA: SRA059446 for ITS and SRA060238 for LSU. OTU distribution table with identification uploaded as online supplemental material.

Supporting information

Additional supporting information may be found in the online version of this article.

Fig. S1 The Gigante fertilization experiment, an NPK factorial fertilization in a lowland Panama rainforest. Plots are 40 × 40 m.

Fig. S2 Modified version of V-Xtractor (Hartmann *et al*. 2010) for use with the nuclear ribosomal large subunit (LSU). (Zip file) Modified version of V-Xtractor (Hartmann et al. 2010) for use with the nuclear ribosomal large subunit (LSU). Tool can be downloaded at: http://www.microbiome.ch/web/Tools.html

Fig. S3 Comparison of ITS OTUs determined at different sequence similarities. Square—Full ITS, circle—ITS1, triangle—ITS with singletons removed, and cross—ITS1 with singletons removed. ITS1 extracted and with singletons removed is the most conservative when determining the number of OTUs. For the purpose of this study, OTUs were conservatively determined at 95% sequence similarity. Arrow shows chosen value for OTU determination.

Fig. S4 Comparison of ITS1 OTU distribution across class (A), order (B) and family (C). The black line separates ascomycetes and basidiomycetes; above the black line represents basidiomycetes and below are ascomycetes.

Fig. S5 NMDS plot using the Jaccard index of ITS1 sequences normalized to 950 sequences per treatment (N, P, K, M and control treatments). The numbers following the treatment represent the replicate of each treatment.

Fig. S6 NMDS plot of ITS1 sequences normalized to 950 sequences per treatment. All treatments included. The numbers following the treatment represent the replicate of each treatment.

Table S1 SAS output of the simplified factorial analysis.

Appendix S1 OTU distribution table with identification..