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Large-scale fungal diversity assessment in the Andean Yungas forests reveals strong community turnover among forest types along an altitudinal gradient

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Abstract

The Yungas, a system of tropical and subtropical montane forests on the eastern slopes of the Andes, are extremely diverse and severely threatened by anthropogenic pressure and climate change. Previous mycological works focused on macrofungi (e.g. agarics, polypores) and mycorrhizae in *Alnus acuminata* forests, while fungal diversity in other parts of the Yungas has remained mostly unexplored. We carried out Ion Torrent sequencing of ITS2 rDNA from soil samples taken at 24 sites along the entire latitudinal extent of the Yungas in Argentina. The sampled sites represent the three altitudinal forest types: the piedmont (400–700 m a.s.l.), montane (700–1500 m a.s.l.) and montane cloud (1500–3000 m a.s.l.) forests. The deep sequence data presented here (i.e. 4 108 126 quality-filtered sequences) indicate that fungal community composition correlates most strongly with elevation, with many fungi showing preference for a certain altitudinal forest type. For example, ectomycorrhizal and root endophytic fungi were most diverse in the montane cloud forests, particularly at sites dominated by *Alnus acuminata*, while the diversity values of various saprobic groups were highest at lower elevations. Despite the strong altitudinal community turnover, fungal diversity was comparable across the different zonal forest types. Besides elevation, soil pH, N, P, and organic matter contents correlated with fungal community structure as well, although most of these variables were co-correlated with elevation. Our data provide an unprecedented insight into the high diversity and spatial distribution of fungi in the Yungas forests.

Keywords: biodiversity, fungi, Ion Torrent sequencing, metabarcoding

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Introduction

Fungi represent one of the largest groups of living organisms. Already well before the routine use of DNA sequencing in fungal diversity assessments, their true diversity was estimated to be around 1.5 million species (Hawksworth 1991), while more recent estimates suggest that there may be 0.7–5 million fungal species (Schmit & Mueller 2007; Blackwell 2011). Although there are differences between various estimates, it is clear that with the approximately 100 000 described species, we currently know only a fraction of the total fungal diversity (Blackwell 2011). Because fungi play central roles in the functioning of terrestrial ecosystems as plant symbionts, pathogens and decomposers, it is necessary to gain adequate taxonomic and ecological knowledge to conserve and utilize biodiversity and to

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recognize and to respond intelligently to recent and future environmental changes (Donoghue *et al.* 2009). The task of discovering a significant portion of yet unknown fungal species has only recently become possible with the advent of high-throughput DNA sequencing of environmental samples. In particular, DNA metabarcoding, that is, the automated identification of multiple species from an environmental sample (Taberlet *et al.* 2012), has greatly advanced our knowledge of fungal diversity already and has enormous potential to further boost data acquisition in biodiversity research (Schadt *et al.* 2003; Geml *et al.* 2009, 2010, 2012; Abarenkov *et al.* 2010; Jumpponen *et al.* 2010; Taylor *et al.* 2010; Tedersoo *et al.* 2010b; Hibbett *et al.* 2011; Brown *et al.* 2013).

Acquiring such knowledge on the diversity, composition and spatial structures of fungal communities is particularly important in undersampled and presumably highly diverse areas, such as the Andean Yungas in South America. The Yungas consist of a system of tropical and subtropical humid montane forests developed on the eastern slopes of the Andes as a result of orographic rains (caused by condensation of humid air rising up high mountain slopes) brought by wet winds from the Atlantic Ocean (Cabrera 1976). The Yungas reach their southern limit in northwestern Argentina, where they form an approximately 700-km-long and <50-km-wide forest belt from the border with Bolivia to Catamarca Province (Fig. 1). These subtropical southern Andean Yungas are also called the 'Selva Tucumano-Boliviana', where flora elements of Holarctic, Gondwanan, Pantropical and Neotropical origins coexist (Grau & Brown 2000; Brown *et al.* 2005). This region is floristically distinct from the tropical northern Andean forests (Brown *et al.* 2001) and constitutes the southern limit of the Amazonian biogeographic domain (Cabrera 1976).

The Argentinian Yungas are divided into three biogeographic sectors: the northern, the central and the southern sectors (Brown *et al.* 2001; Fig. 1). Available data on the distribution of many plant and animal species indicate that species diversity decreases with increasing latitude, and thus, the northern sector is considered the richest in species (Ojeda & Mares 1989; Brown 1995; Morales *et al.* 1995; Brown *et al.* 2001, 2005). Within the Yungas, when compared to this latitudinal trend, the strong altitudinal and the resulting environmental gradients have a much more profound effect on the species distribution and community composition of plants and animals (Brown *et al.* 2001; Malizia *et al.* 2012). For example, forests at lower elevations are characterized by plants capable of withstanding prolonged dry periods and temperatures above 40°C, while species adapted to high humidity and lower temperatures (including occasional frosts and snow storms) are found at higher elevations (Brown *et al.* 2001). Therefore, the Yungas are classified into three major altitudinal forest types: the piedmont forest (400–700 m a.s.l.), the montane forest (700–1500 m a.s.l.) and the montane cloud forest (1500–3000 m a.s.l.) (Brown *et al.* 2001, 2005). In Argentina, the flora and fauna of the Yungas have been relatively well studied and are considered very diverse and rich in endemics (Meyer 1963; Hueck 1978; Legname 1982; Ojeda & Mares 1989; Brown 1990; Lavilla & Manzano 1995; Blake & Rougés 1997). The Yungas and the subtropical humid forests in northeastern Argentina ('Selva Paranaense') cover <2% of Argentina's area, yet they harbour more than 50% of the country's biodiversity (Brown *et al.* 1993). Unfortunately, the Yungas, particularly the piedmont forests, have been severely affected by agriculture and are further threatened by anthropogenic pressure and climate change (Grau & Brown 2000; Brown *et al.* 2002; Pacheco *et al.* 2010; Malizia *et al.* 2012).

Early extensive mycological explorations of Argentina, including various regions from Patagonia to the Yungas, were carried out by Spegazzini (Spegazzini 1912, 1919) and Singer (Singer 1953; Singer & Morello 1960), with a focus on macrofungi. More recent mycological works in the Yungas dealt with mycorrhizal fungi associated with *Alnus acuminata* in the montane cloud forests (Becerra *et al.* 2002, 2005a,b, 2007, 2009a, 2011; Nouhra *et al.* 2005; Pritsch *et al.* 2010), and the systematics and distribution of various groups of macrofungi (Hladki & Romero 2001, 2010; Robledo *et al.* 2003; Urcelay & Robledo 2004; Robledo & Rajchenberg 2007; Robledo 2009; Baroni *et al.* 2012; Niveiro 2013). Despite these important contributions, most of the fungal diversity in the Yungas has remained unexplored, particularly with respect to 'microfungi', such as ascomycetes and the early diverging fungal lineages. In this study, we sampled multiple sites representing the three major forest types and conducted the first large-scale diversity assessment of soil fungi in the Yungas using DNA metabarcoding. The aims of this work were to characterize fungal communities in the three altitudinal forest types along the geographic range of the Yungas in Argentina and to assess correlations between environmental variables and fungal community composition.

Materials and methods

The study region

Soil samples were collected in early May in 2011 and 2013 in Jujuy, Salta and Tucumán provinces in Argentina. The 24 sampling sites represent the entire latitudinal extent of the Yungas in Argentina (approximately S 22–28°) as well as all the three altitudinal zones

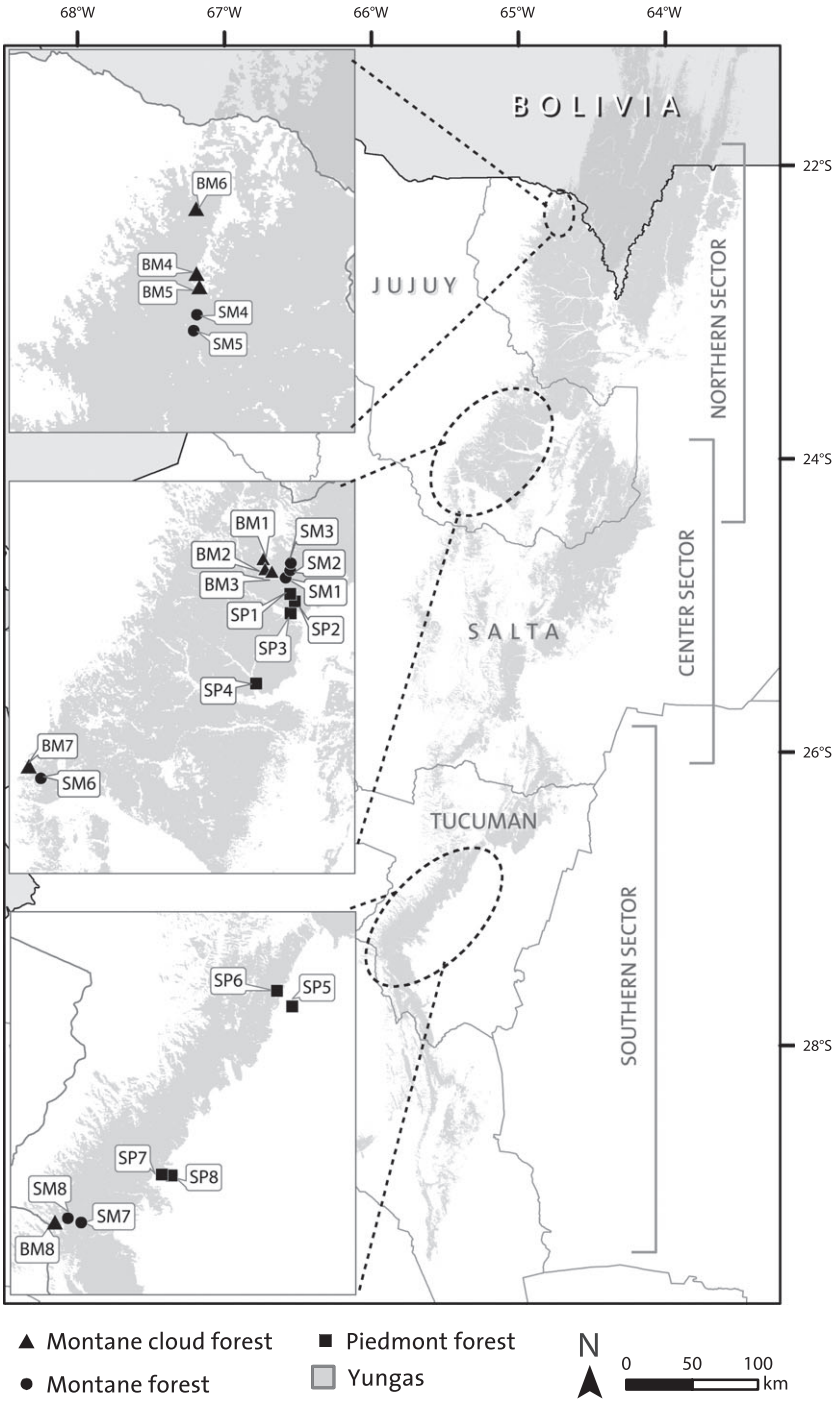


Fig. 1 A map of northwestern Argentina, showing the geographic distribution of the Andean Yungas forests with the sampling locations indicated.

described below, with eight sampling localities in each zone. Forest type, elevation, geographic coordinates, locality, and the measured soil chemical properties of these locations are shown in Table 1. All sampling sites in Salta and Jujuy provinces are part of The Yungas UNESCO Biosphere Reserve (Lomáscolo *et al.* 2010).

Piedmont forests cover the foothills up to approximately 700 m a.s.l. and can be considered a transitional zone between the xerophytic forest of the Occidental Chaco and the more humid montane forest (Cabrera 1976). The precipitation ranges from 600 to 1000 mm and is markedly seasonal, with approximately 80% falling in the wet season (November to March) (Vervoorst 1982; Brown 1995). This seasonality is also reflected in the proportion of deciduous species (79%) that is among the highest in all South American forests (Brown

Table 1 Sampling sites of various Yungas forest types included in this study with code, vegetation type, elevation, geographic coordinates, locality, and some of measured soil chemical properties: pH, organic matter (OM), nitrogen (N), and phosphorus (P)

Site	Vegetation type	Elevation (m a.s.l.)	Latitude, longitude	Locality	Province	pH	OM (%)	N (%)	P (ppm)
BM1	Montane cloud forest	1690–1720	−23.6823; −64.9019	Parque Nacional Calilegua	Jujuy	4.36	10.10	0.45	54.46
BM2	Montane cloud forest	1650–1670	−23.6853; −64.8997	Parque Nacional Calilegua	Jujuy	3.75	11.69	0.48	38.42
BM3	Montane cloud forest	1540–1570	−23.6847; −64.8910	Parque Nacional Calilegua	Jujuy	5.81	4.32	0.19	234.70
BM4	Montane cloud forest	1690–1720	−22.3424; −64.7256	Vicinity of Los Toldos	Salta	4.26	9.97	0.49	2.94
BM5	Montane cloud forest	1723–1735	−22.3556; −64.7225	Vicinity of Los Toldos	Salta	4.60	12.89	0.63	1.80
BM6	Montane cloud forest	1752–1756	−22.2778; −64.7252	Reserva Nacional El Nogalar	Salta	4.86	12.36	0.63	6.71
BM7	Montane cloud forest	2150–2160	−24.0937; −65.4827	Parque Provincial Potrero de Yala	Jujuy	4.44	9.83	0.46	10.49
BM8	Montane cloud forest	1820–1905	−27.3309; −65.9513	Vicinity of Parque Nacional Campo de Los Alisos	Tucumán	5.26	8.45	0.41	3.60
SM1	Montane forest	1050–1120	−23.7050; −64.8659	Parque Nacional Calilegua	Jujuy	5.94	6.44	0.29	162.04
SM2	Montane forest	920–940	−23.6966; −64.8617	Parque Nacional Calilegua	Jujuy	6.23	5.71	0.27	88.43
SM3	Montane forest	1010–1020	−23.6948; −64.8636	Parque Nacional Calilegua	Jujuy	6.38	6.24	0.27	18.60
SM4	Montane forest	1642–1648	−22.3833; −64.7257	Vicinity of Parque Nacional Baritú	Salta	4.77	11.69	0.55	7.66
SM5	Montane forest	1610–1618	−22.3990; −64.7293	Vicinity of Parque Nacional Baritú	Salta	5.43	12.09	0.57	10.58
SM6	Montane forest	1668–1682	−24.1206; −65.4541	Parque Provincial Potrero de Yala	Jujuy	5.75	12.89	0.66	29.45
SM7	Montane forest	915–920	−27.3335; −65.8781	Vicinity of Parque Nacional Campo de Los Alisos	Tucumán	6.01	9.30	0.43	13.32
SM8	Montane forest	1348–1352	−27.3226; −65.9147	Vicinity of Parque Nacional Campo de Los Alisos	Tucumán	6.24	7.04	0.36	4.73
SP1	Piedmont forest	550	−23.7571; −64.8503	Parque Nacional Calilegua	Jujuy	6.85	6.31	0.27	121.46
SP2	Piedmont forest	570–600	−23.7522; −64.8510	Parque Nacional Calilegua	Jujuy	7.17	4.78	0.25	79.94
SP3	Piedmont forest	620–650	−23.7554; −64.8534	Parque Nacional Calilegua	Jujuy	5.73	5.65	0.29	108.25
SP4	Piedmont forest	519–526	−23.9188; −64.9345	Parque Nacional Calilegua	Jujuy	6.76	8.11	0.38	60.22
SP5	Piedmont forest	491	−26.8091; −65.2830	Parque Percy Hill	Tucumán	6.35	8.29	0.38	70.00
SP6	Piedmont forest	743–775	−26.7701; −65.3251	Reserva Provincial Sierra de San Javier	Tucumán	5.99	9.83	0.47	38.42
SP7	Piedmont forest	405	−27.2218; −65.6256	Reserva Provincial La Florida	Tucumán	6.11	6.51	0.32	40.87
SP8	Piedmont forest	431	−27.2198; −65.6318	Reserva Provincial La Florida	Tucumán	6.33	7.18	0.38	56.25

et al. 2001). The characteristic natural community in these piedmont forests is dominated by *Calycophyllum multiflorum* (Rubiaceae), *Phyllostilon rhamnoides* (Rhamnaceae), *Handroanthus impetiginosus* (Bignoniaceae), *Anadenanthera colubrina*, *Myroxylon peruiferum* (Leguminosae), *Cordia americana*, *C. trichotoma* (Boraginaceae), and *Astronium urundeuva* (Anarcardiaceae). The tree crowns reach an average height of approximately 30 m, and there is a fairly dense shrub layer (approximately 2 m high), as well as many lianas, climbers and epiphytes (Brown *et al.* 2001).

Montane forests, considered the most species-rich of the three forest zones for plants and animals, occupy the slopes between 700 and 1500 m a.s.l. The precipitation is also the highest in this zone, ranging from 1000 to more than 2000 mm. In general, the montane forests are composed of perennifolious species of tropical origin that reach the southern limit of their distribution here, for example *Ficus maroma* (Moraceae), *Cinnamomum porphyrium*, *Nectandra cuspidata*, *Ocotea puberula* (Lauraceae), *Inga edulis*, *I. marginata*, *I. saltensis*, *Tipuana tipu* (Leguminosae), and *Blepharocalix salicifolius* (Myrtaceae) (Brown *et al.* 2001). Although lianas, climbers, and shrubs are less abundant here than in the piedmont forest, the epiphytes (orchids, bromeliads, cacti, ferns, etc.) reach their greatest diversity and abundance in this

zone, particularly between 1000 and 1500 m, where more than 70% of the trees host vascular epiphytes (Brown 1990).

Montane cloud forests, which inhabit slopes above 1500 m a.s.l., represent the uppermost forest zone of the Yungas and, at their upper limit, form a heterogeneous ecotone with the high grasslands. The annual rainfall ranges from 1000 to 1500 mm, supplemented by year-round fog providing additional precipitation that is estimated to be equivalent to rainfall. The common species in this zone have typically Andean distributions, including species with Gondwanan as well as Holarctic origins. Examples for the Gondwanan group include *Podocarpus parlatorei* (Podocarpaceae), *Roupala* sp. (Proteaceae), *Gunnera* sp. (Gunneraceae), and *Fuchsia boliviana* (Onagraceae), while species with Holarctic origins are *Alnus acuminata* (Betulaceae), *Juglans australis* (Juglandaceae), *Viburnum seemenii*, *Sambucus nigra* (Caprifoliaceae), and *Ilex argentinum* (Aquifoliaceae) (Brown *et al.* 2001). Due to the high humidity, epiphytic lichens, mosses, and ferns are very abundant in this zone (J. Geml pers. obs.).

Sampling and molecular work

At each site, 40 soil cores, 2 cm in diameter and approximately 20 cm deep, were taken in a way that cores were at least 2 m from each other to minimize the probability of sampling the same genet repeatedly. The 40 cores were pooled, resulting in a composite soil sample for each site. Because freezing the samples was not feasible due to the remoteness of some of the sampling locations, approximately 10 g of each composite soil sample was deposited in a sealed plastic bag and was dried in the field using silica gel for the DNA work. The remaining parts of the pooled samples were kept for soil chemical analyses that were later carried out at the Laboratorio de Suelos y Aguas of the Facultad de Ciencias Agropecuarias (Universidad Nacional de Córdoba) following protocols described in Sparks *et al.* (1996).

Genomic DNA was extracted from 1 g of dry soil using NucleoSpin® Soil kit (Macherey-Nagel Gmbh & Co., Düren, Germany), according to the manufacturer's protocol. For each sample, DNA extraction was carried out twice and replicates were combined. One microlitre of DNA template was used for the 40 μL PCR containing 25.6 μL of MQ water, 4 μL of 10× buffer, 1.5 μL dNTPs (2.5 mM), 1.5 μL of reverse and forward primers (10 mM), 4 μL $MgCl_2$ (50 mM), 0.5 μL BSA (10 mg/mL) and 0.4 μL BIOTAQ polymerase (5 U/μL). Primers fITS7 (Ihrmark *et al.* 2012) and ITS4 (White *et al.* 1990) with Ion Torrent adapters were used to amplify the ITS2 region (approximately 250 bp) of the nuclear ribosomal rDNA repeat, using the following PCR conditions: one cycle of 95°C for 5 min; then 37 cycles of 95°C for 20 s, 56°C for 30 s and 72°C for 1.5 min; and ending with one cycle of 72°C for 7 min. ITS is the universal DNA barcode marker for fungi and has been used in a wide variety of taxonomic and ecological studies (Bruns *et al.* 1991; Geml *et al.* 2005; O'Brien *et al.* 2005; Orihara *et al.* 2012; Schoch *et al.* 2012; Gomes *et al.* 2013; Walther *et al.* 2013). The ITS4 primer was labelled with sample-specific multiplex identification DNA-tags (MIDs). Ion Torrent adaptors, primers and MIDs used in this study are listed in Table S1 (Supporting information). A negative control consisting of MQ water instead of DNA was made and underwent the PCR under the same experimental conditions and was shown on a gel to be amplicon free. The PCR products were assessed for size distribution and for DNA concentration using a Bioanalyser 2100 (Agilent Technologies Inc., Santa Clara, CA, USA) and were cleaned up using 0.9× Ampure® beads (Beckman Coulter, Beverly, MA, USA) to remove short fragments. The amplicons were diluted with MQ water to achieve equal DNA concentration for each sample in the final pool to be sequenced. Two hundred and fifty microlitres of the pool was used for emulsion PCR according to the Ion PGM™ 200Xpress™ Template Kit manual. The amplicon library was sequenced using an Ion 318™ Chip by an Ion Torrent Personal Genome Machine (PGM; Life Technologies, Guilford, CT, USA) at the Naturalis Biodiversity Center.

Bioinformatic work

The raw sequence data contained 7 489 045 sequences with a modal read length of 287. The initial clean-up of the sequence data was carried out using the online platform Galaxy (https://main.g2.bx.psu.edu/root), in which the raw data were converted to FASTQ format using FASTQ Groomer (Blankenberg *et al.* 2010) and filtered by quality (>95% of bases with Q20). Sequences of primers and adapters (identification tags) were removed using FASTX-toolkit. We used a parallel version of MOTHUR v. 1.32.1 (Schloss *et al.* 2009) installed at the University of Alaska Life Sciences Informatics Portal for sequence analyses. Established pipelines for next-generation sequencing data analyses have two main methods for quality filtering: (i) by correcting flowgrams of platform-generated data using PyroNoise (Quince *et al.* 2011) or similar expectation-maximization algorithms and (ii) by implementing a minimum quality threshold of average Phred scores for the entire sequence (e.g. as implemented in the Ribosomal Database Project) or by utilizing a sliding window (e.g. MOTHUR standard protocol). We filtered sequences by quality score with a sliding window approach as in Brown *et al.* (2013). Sequences were

subjected to quality filtering whereby each sequence was screened for thresholds for average Phred score of Q≥25 in a sliding window of 50 bp (qwindowaverage = 25, qwindowsize = 50), no ambiguous bases (maxambig = 0) and homopolymers no longer than 8 bp (maxhomop = 8). Sequences shorter than 150 bp or longer than 400 bp were omitted from further analyses (minlength = 150, maxlength = 400). A total of 4 108 126 sequences remained after quality filtering and trimming with an average read length of 257.4 ± 55.4 (mean ± SD). Because next-generation sequencing libraries generally vary in size, we normalized the number of sequences for all samples prior to OTU clustering, as recommended by Gihring *et al.* (2012), to ensure that estimators across all samples are comparable. For this purpose, we randomly subsampled the number of trimmed and quality-filtered reads to the size of the smallest library (51 264).

The resulting 1 230 336 sequences served as input for operational taxonomic unit (OTU) clustering. Although there is no universal cut-off value for species delimitation in fungi due to a substantial variability in nucleotide substitution rates and ages of species across fungal lineages, it has been shown that 2–3% ITS sequence divergence usually delimits different species in many basidiomycete lineages (Hughes *et al.* 2009) and a 97% sequence similarity cut-off value is routinely used in fungal community studies (O'Brien *et al.* 2005; Bjorbækmo *et al.* 2010; Bellemain *et al.* 2013; Brown *et al.* 2013; Lindahl *et al.* 2013). Therefore, we clustered the quality-filtered sequences into OTUs based on 97% sequence similarity using OTUPIPE (Edgar *et al.* 2011) while simultaneously removing 248 064 putatively chimeric sequences using a curated data set of fungal ITS sequences of Nilsson *et al.* (2011) as reference data set. Representative sequences of the OTUs were subjected to two independent similarity searches. At first, we compared OTU sequences using USEARCH (Edgar 2010) against the quality-checked UNITE+INSD fungal ITS sequence database containing both identified and unidentified sequences (version released on 15 October 2013), many of which are assigned to species hypothesis groups as defined by experts using phylogenetic evaluations (Kõljalg *et al.* 2013). OTUs that did not have at least 80% similarity over at least 150 bp to any fungal sequence in this UNITE+INSD database were excluded from further analyses. Subsequently, we carried out a BLASTN 2.2.21 (Altschul *et al.* 1997) search against a database containing only identified fungi (Abarenkov *et al.* 2010) to reveal more about the taxonomic affinities of those OTUs that were most similar to unidentified fungal sequences in the first search. Because of the very high number of sequences generated per sample and because most singletons in next-generation sequencing data sets tend to be artifactual and can overestimate the diversity of 'rare taxa' (Kunin *et al.* 2010; Tedersoo *et al.* 2010b), we opted to be conservative and excluded all global singletons (OTUs that were found only once across all samples despite the deep sequencing effort) from further analyses.

Fungal diversity

We calculated Good's coverage (complement of the ratio between local singletons and the total sequence count) for each sample to estimate the exhaustiveness of our deep sequencing efforts. The effect of forest type on observed richness of OTUs (S), Good's coverage estimators, Shannon's diversity (H) and evenness (H/lnS) was tested across all sites using analysis of variance in R (Faraway 2002). In addition, we calculated the species–area curve of all fungal OTUs vs. the number of sampled sites with first- and second-order jackknife to estimate the total fungal diversity in the Yungas. Beta diversity was calculated following Whitaker (1972), that is, $\beta = S_c/S - 1$, where S_c is the total number of OTUs in all samples and S is the average number of OTUs per sample. Moreover, we visualized the distribution of OTUs among the three altitudinal forest types in a Venn diagram using BioVenn (Hulsen *et al.* 2008). Taxonomic classifications were based on Index Fungorum as featured in UNITE.

Comparing fungal communities among sampling sites

We used PC-ORD v. 5.32 (McCune & Grace 2002) to run nonmetric multidimensional scaling (NMDS) on a primary presence/absence matrix of sites by OTUs and a secondary matrix of sites by environmental and fungal community variables (Table 2). Given the very high sequencing coverage we achieved, 'presence' was defined as ≥5 sequences on a per-sample basis following the recommendations of Lindahl *et al.* (2013) to minimize false positives (e.g. OTUs that are common in one sample, but may be low-abundant contaminants in others). The resulting matrix contained 7465 OTUs and was used as input for ordinations. Data were subjected to 500 iterations per run using the Sørensen similarity (Bray–Curtis index) and a random number to start. The solution with the lowest stress was derived from 250 runs using real data and was then subjected to 250 randomized runs using a Monte Carlo test to evaluate the probability of the final NMDS pattern being greater than chance occurrences. The solution with the lowest number of dimensions was selected when the decrease in the final stress was <5 by adding another dimension (McCune & Grace 2002). The Pearson correlation coefficient (r) values between environmental and fungal community variables and axes 1 and 2 were calculated.

Table 2 Pearson's correlation values (*r*) for variables in the NMDS ordination performed with the OTU vs. site matrix, with numbers of OTUs given for the taxonomic groups. The data set was a presence–absence matrix, where 'presence' was defined as ≥5 sequences on a per-sample basis, and contained 7465 OTUs. Final stress value for the two-dimensional NMDS solution was 0.07114. Variables with $|r| \geq 0.5$ values are shown in bold and are displayed in the NMDS ordinations in Fig. 4

		r-value				*r*-value				*r*-value	
	OTUs	Axis 1	Axis 2		OTUs	Axis 1	Axis 2		OTUs	Axis 1	Axis 2
Environmental variables				*Orders of Ascomycota*				*Orders of Basidiomycota*			
pH		−0.883	0.045	**Archaeorhizomycetales**	18	0.647	−0.127	**Agaricales**	971	−0.596	−0.712
Organic matter		0.678	0.344	**Botryosphaeriales**	24	−0.5	0.003	Agaricostilbales	3	n.a.	n.a.
N		0.634	0.425	Capnodiales	68	−0.44	−0.181	Atheliales	5	n.a.	n.a.
C:N		0.064	−0.51	Chaetosphaeriales	31	0.31	0.491	Atractiellales	7	−0.214	0.196
P		−0.496	−0.532	**Chaetothyriales**	250	−0.06	−0.521	**Auriculariales**	16	−0.391	−0.724
Elevation		0.87	−0.051	Coniochaetales	23	0.425	0.251	Boletales	9	n.a.	n.a.
Total OTUs	7465	−0.433	−0.66	Diaporthales	28	−0.498	−0.327	**Cantharellales**	90	−0.506	−0.632
Latitude		−0.124	0.502	Dothideales	11	n.a.	n.a.	Corticiales	9	−0.15	−0.106
				Eurotiales	144	−0.099	0.589	Cystofilobasidiales	8	0.136	−0.252
				Geoglossales	28	0.169	−0.336	Dacrymycetales	2	n.a.	n.a.
				Helotiales	345	0.906	−0.028	Entylomatales	2	n.a.	n.a.
Fungal phyla and ecological groups				**Hypocreales**	600	−0.608	0.362	Filobasidiales	5	0.35	−0.018
Ascomycota	3252	−0.438	−0.273	Hysteriales	1	n.a.	n.a.	**Geastrales**	112	−0.72	−0.544
Basidiomycota	1904	−0.357	−0.783	Jahnulales	1	n.a.	n.a.	Geminibasidiales	1	n.a.	n.a.
Chytridiomycota	37	0.186	−0.125	Lecanorales	2	n.a.	n.a.	Gomphales	1	n.a.	n.a.
Glomeromycota	135	−0.606	−0.372	Lecideales	4	n.a.	n.a.	**Hymenochaetales**	27	−0.209	−0.506
Zygomycota	371	0.411	−0.277	**Leotiales**	20	0.831	−0.101	Hysterangiales	2	n.a.	n.a.
DSE *s.s.*	85	0.654	−0.033	Lulworthiales	3	n.a.	n.a.	**Leucosporidiales**	9	0.718	0.313
ECM *s.s.*	130	0.678	−0.118	**Microascales**	10	−0.553	−0.152	**Malasseziales**	6	−0.383	−0.606
ECM/DSE	51	0.628	0.232	Myriangiales	1	n.a.	n.a.	Microbotryales	1	n.a.	n.a.
				Onygenales	25	−0.625	−0.148	Microstromatales	2	n.a.	n.a.
				Orbiliales	3	n.a.	n.a.	Polyporales	64	−0.424	−0.314
				Ostropales	1	n.a.	n.a.	**Russulales**	37	0.747	0.064
				Patellariales	6	n.a.	n.a.	**Sebacinales**	47	0.504	−0.407
				Peltigerales	17	−0.339	−0.667	Sporidiobolales	63	−0.118	−0.08
				Pezizales	61	−0.372	−0.426	Thelephorales	75	0.392	−0.42
				Phyllachorales	3	n.a.	n.a.	Trechisporales	18	0.467	−0.307
				Pleosporales	384	−0.528	−0.437	Tremellales	121	0.478	−0.005
				Saccharomycetales	26	−0.213	−0.057	**Trichosporonales**	29	0.303	0.719
				Sordariales	146	−0.297	−0.108	Wallemiales	6	−0.399	−0.202
				Teloschistales	3	n.a.	n.a.	Incertae sedis	11	n.a.	n.a.

Table 2 *Continued*

	OTUs	*r*-value Axis 1	*r*-value Axis 2
Thelebolales	15	0.656	−0.319
Trichosphaeriales	4	−0.508	0.44
Umbilicariales	1	n.a.	n.a.
Verrucariales	12	n.a.	n.a.
Xylariales	236	−0.733	−0.265
Incertae sedis	326	n.a.	n.a.
unidentified	371	n.a.	n.a.
unidentified	145	n.a.	n.a.

ECM *s.s.*, ectomycorrhizal fungi *sensu stricto* based on Tedersoo *et al.* (2010a); DSE *s.s.*, dark septate endophytes *sensu stricto* based on Grünig *et al.* (2011); ECM/DSE, ascomycetes that are capable of forming both ECM and DSE symbioses. Pearson's correlation values were not calculated for taxonomic orders with <5 OTUs in any site and are indicated with 'n.a.'.

Orthogonal rotation of the resulting NMDS solution was used to maximize correlation between the strongest environmental variables (as indicated by Pearson *r* values) and major axes. Relationships between variables were estimated using regression analysis implemented in Microsoft Excel. In addition to the edaphic factors and the various taxonomic groups, we also calculated the correlation of the number of OTUs representing root-associated fungal groups, that is, ectomycorrhizal (ECM) and dark septate endophytic (DSE) fungi based on lists of known ECM- and DSE-forming taxa published by Tedersoo *et al.* (2010a) and Grünig *et al.* (2011), respectively. We also tested whether fungal communities were statistically different across forest types using two methods: multiresponse permutation procedure (MRPP) and permutation-based nonparametric MANOVA (Anderson 2001). Subsequently, we performed Mantel tests in PC-ORD to test whether community structure is correlated with geographic (spatial) distance and/or with the environmental conditions. For the latter, mean and standard deviation values of the environmental variables were standardized so that all variables could have the same influence on the data. Finally, we determined any preferences of individual OTUs for specific altitudinal forest types using indicator species analyses, also in PC-ORD.

Results

Fungal diversity

Of the 7 489 045 original sequences, 4 108 126 passed the series of quality-filtering steps. After normalizing the library size across all samples, 1 230 336 sequences were assembled into 14 120 nonsingleton OTUs and 13 248 singletons. After excluding OTUs with <80% similarity or <150 bp pairwise alignment length to a fungal sequence as performed by USEARCH, 14 039 OTUs were retained. The observed number of OTUs did not differ significantly among the three altitudinal zones ($F = 0.05$, $P = 0.96$; Fig. 2a), and we observed practically no correlation between altitude and OTU richness ($r = -0.13938$). Good's coverage estimators (0.980 ± 0.006, mean $\pm$ SD across all sites) indicate that the deep sequencing allowed for a very high OTU coverage. The coverage estimators did not differ among the three altitudinal zones ($F = 0.03$, $P = 0.97$), suggesting that our sequencing effort was similarly deep across all sites (Fig. 2b). Similarly, there was no significant difference between Shannon's diversity index ($F = 0.26$, $P = 0.78$) and evenness values ($F = 0.31$, $P = 0.73$) among the forest types (Fig. 2c,d). The species–area curve generated based on the accumulating number of OTUs with increased number of sites indicated that the

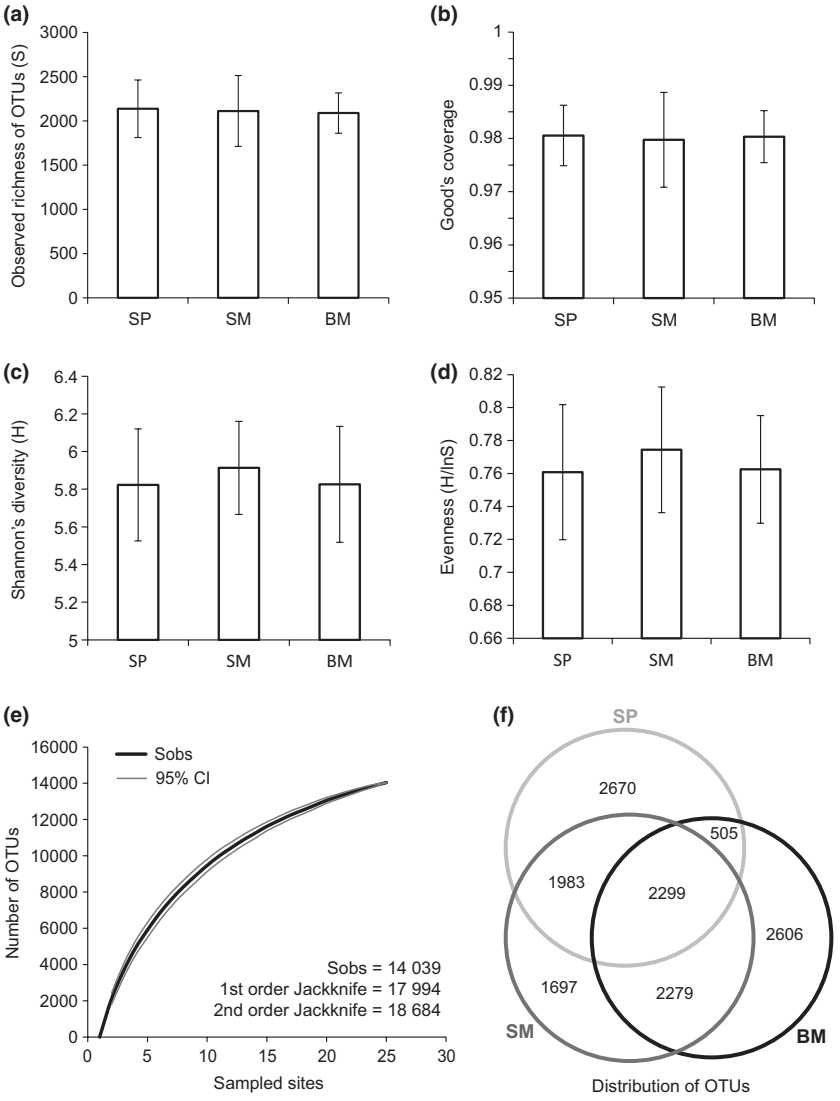


Fig. 2 Community richness and coverage estimators across the sampled locations: (a) observed number of OTUs (S) in the three altitudinal forest types with standard deviations, (b) Good's coverage in the three altitudinal forest types with standard deviations, (c) Shannon's diversity index (H), (d) evenness (H/lnS), (e) rarefaction curve of the total number of fungal OTUs with 95% confidence interval and with first- and second-order jackknife estimates of OTU richness in the Yungas and f) area-proportional Venn diagram comparing OTU richness across the sampled forest types. The size of each sample component is based on the total number of OTUs for that sample relative to the other samples. SP, piedmont forest; SM, montane forest; BM, montane cloud forest.

majority of the soil fungi that occur in the Yungas probably have been sampled in our study, with 14 039 observed OTUs and first- and second-order jackknife estimates of 17 994 and 18 684, respectively (Fig. 2e). It is worth to note that many sequences were excluded from further analyses via the normalization process that was inevitable to make the samples comparable among sites. This probably resulted in the exclusion of some rare OTUs, and the total diversity of fungi in the Yungas probably is even higher than our estimates above. Beta diversity values were high for entire data set (5.648) and medium within the altitudinal zones (SP: 2.491, SM: 2.911 and BM: 2.683). The Venn diagram indicated that half (49.67%) of the OTUs occurred in only one forest type, suggesting high community turnover among the altitudinal zones, and that the mid-elevation montane forests shared four times as many OTUs with either piedmont or montane cloud forest zones than these latter shared with each other (Fig. 2f).

Of the 14 039 fungal OTUs, Ascomycota was by far the dominant phylum and accounted for 6355 OTUs (45.27%), followed by Basidiomycota with 3511 (25.01%). Glomeromycota was represented by 409 OTUs (2.91%), while basal lineages formerly classified in Zygomycota accounted for 723 OTUs (5.15%) and Chytridiomycota for 67 OTUs (0.48%). In addition, there were 2974 (21.18%) unidentified fungal OTUs with most similar sequences to other environmental sequences without assignment to a phylum. The top 200 OTUs by sequence count and the corresponding most similar publicly available sequences are shown in Table S2 (Supporting information). In Ascomycota, there were several taxonomic orders with a high number of OTUs, such as Hypocreales, Pleosporales, Helotiales, Chaetothyriales, and Xylariales, while in Basidiomycota, the

order Agaricales was by far the most diverse, accounting for more than half of all basidiomycete OTUs. The number and proportional distribution of OTUs representing all detected taxonomic phyla and orders are shown in Fig. 3.

Comparing fungal communities among sampling sites

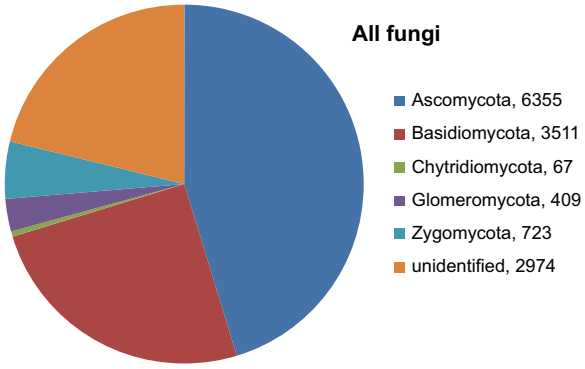
Nonmetric multidimensional scaling analyses resulted in a two-dimensional solution with a final stress of 0.07144 and a final instability <0.00001. The Monte Carlo test results indicated that this two-dimensional solution using the real data was significantly better than chance occurrences ($P = 0.004$). The two axes explained the majority of variability in the sampled fungal communities (axis 1: $r^2 = 0.700$; axis 2: $r^2 = 0.198$; total $r^2 = 0.898$; orthogonality = 94.9%). The NMDS ordination plot was orthogonally rotated by elevation. The Pearson correlation coefficient (r) values between all environmental and fungal community variables and axes 1 and 2 are displayed in Table 2. Following Rogers *et al.* (2009), variables with $|r| \geq 0.5$ values for either axis were considered important for characterizing changes in fungal community structure and were superimposed on the ordination plot as direction and strength vectors (Fig. 4).

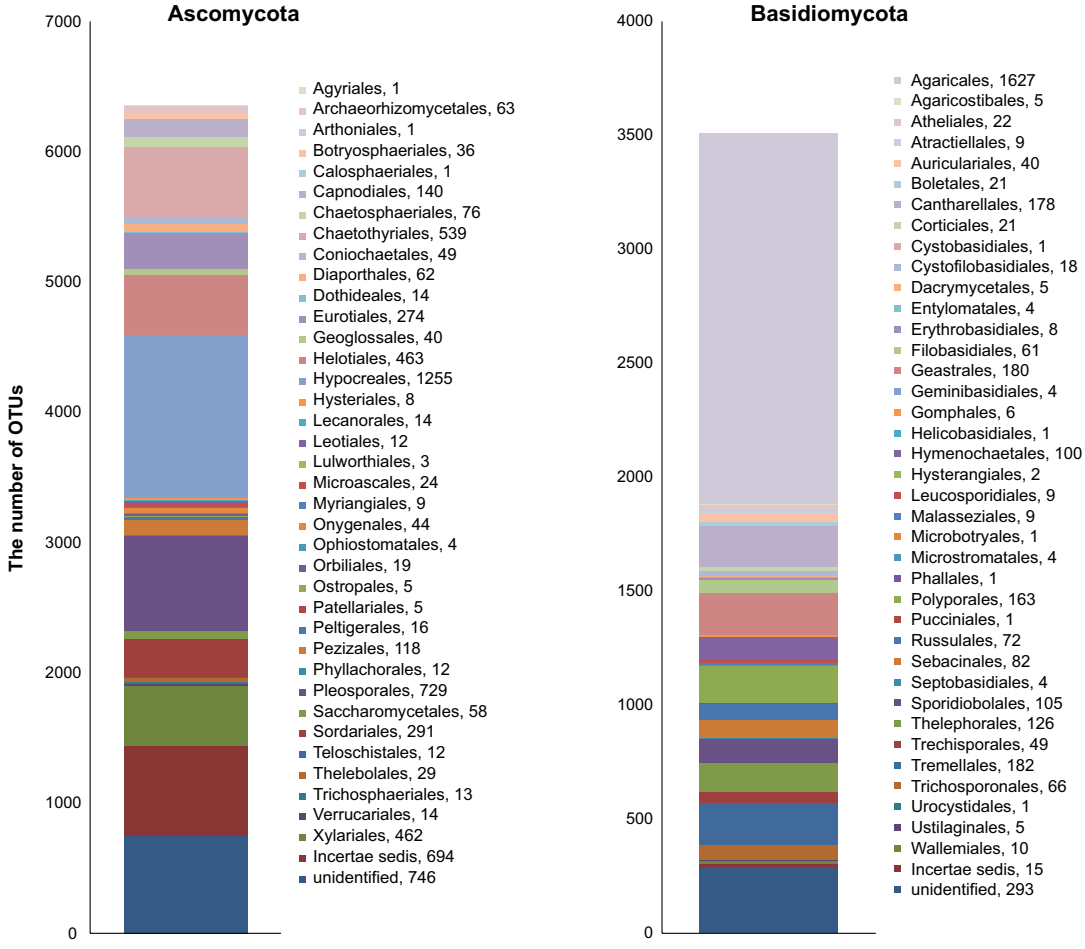
The NMDS plot revealed a strong structuring of fungal communities according to altitudinal forest types and sampling sites representing the same forest type grouped together (Fig. 4). Mantel tests showed that fungal community structure was primarily explained by environmental variables ($r^2 = 0.3025$; $P = 0.00063$) and not by geographic distance ($r^2 = 0.0154$; $P > 0.05$). MRPP confirmed the importance of altitudinal forest types in shaping fungal community composition (effect size $A = 0.07959$, probability $P = 0.00002$). Similarly, permutation-based MANOVA indicated that fungal community structure differed significantly among the altitudinal zones both in the full data set ($P = 0.0002$) and in all pairwise comparisons (all $P < 0.05$). The regression analyses showed that elevation was negatively correlated with soil pH ($r = -0.8052$) and was positively correlated with soil organic matter ($r = 0.6143$) and nitrogen content ($r = 0.5820$). These relationships were also evident in the NMDS plot. Latitude was positively correlated with axis 2, with negative correlations with the total number of OTUs, phosphorus and the C/N ratio (Table 2, Table S3, Supporting information). Both MRPP and the regression analysis indicated a relatively weak, but significant negative correlation between latitude and OTU richness ($A = 0.07481$, $P = 0.00079$, $r = -0.4777$). By analysing the three altitudinal zones separately, we observed the strongest correlation between latitude and OTU richness in the piedmont forest ($r = -0.8029$), followed by the montane cloud forest ($r = -0.5629$), while the latitudinal trend was very weak in the mid-elevation montane forest ($r = -0.3772$).

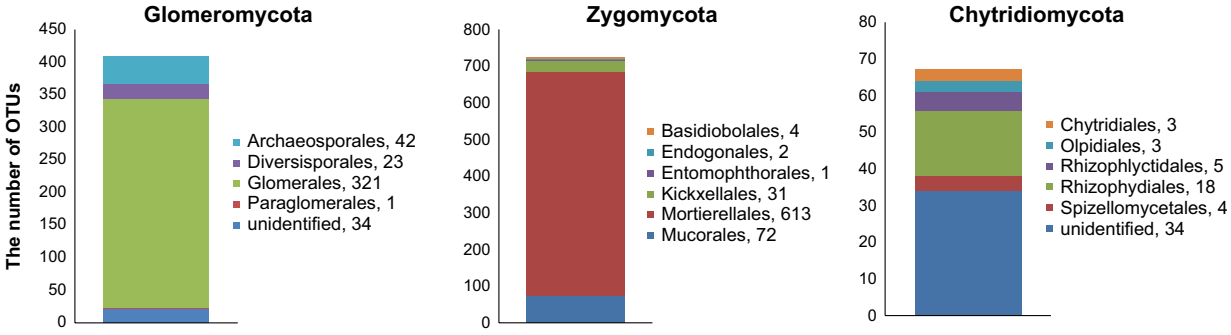
Among the various fungal groups, OTU richness of ECM and DSE fungi was the greatest in the high elevation sites, particularly in those dominated by *Alnus acuminata*. While alder-associated ECM fungi were diverse in the montane cloud forests, we also detected several unidentified Thelephoraceae OTUs in the montane forest and piedmont zones and their closest known relatives (with INSD accession numbers given) had been sequenced from roots of Neotropical Nyctaginaceae hosts: AY667420, Ecuador, *Guapira* sp.; JX548262, Puerto Rico, *Guapira fragrans*; and JX548274–JX548276, Puerto Rico, *Pisonia taina*. At the phylum level, Glomeromycota was negatively correlated with axis 1 (elevation), being more diverse at lower elevations, while Basidiomycota showed a strong negative correlation with axis 2. Among the taxonomic orders, Helotiales and Leotiales in Ascomycota and Leucosporidiales and Russulales in Basidiomycota showed the strongest positive correlation with axis 1, while strong negative correlation was observed in Xylariales, Onygenales, Hypocreales in Ascomycota and by Agaricales and Geastrales in Basidiomycota, among others (Table 2, Fig. 4).

Indicator species analyses found 208 OTUs as significant ($P < 0.05$) indicators for a certain altitudinal forest type. Of these, 112 were indicators for the montane cloud forest (BM), 9 for the montane forest (SM) and 87 for the piedmont forest (SP). We tentatively identified 133 indicator OTUs to species or species complexes based on >97% sequence similarity to a reference sequence. Numerous indicators of the high-elevation montane cloud forest belonged to root-associated fungi, such as ECM (e.g. *Alnicola* sp., *A. subconspersa*, *Cortinarius* sp., *C. helvelloides*, *Inocybe* sp., *I. jacobi*, *Lactarius* sp., *Thelephora alnii*, *Tomentella* sp., *T. ellisii*, *T. sublilacina* and *T. testaceogilva*) and root endophytes (*Chloridium virescens*, *Cladophialophora* sp., *Ilyonectria radicicola*, etc.) as well as several unidentified Helotiales, an order rich in both ECM and DSE fungi (Tedersoo *et al.* 2009a). By contrast, most indicator species at lower elevations were

Fig. 3 The number and proportional distribution of the 14 039 fungal 97% ITS sequence similarity OTUs representing all detected taxonomic phyla and orders in our samples. Assignment to phyla and taxonomic orders was based on closely related sequences found in the UNITE database.







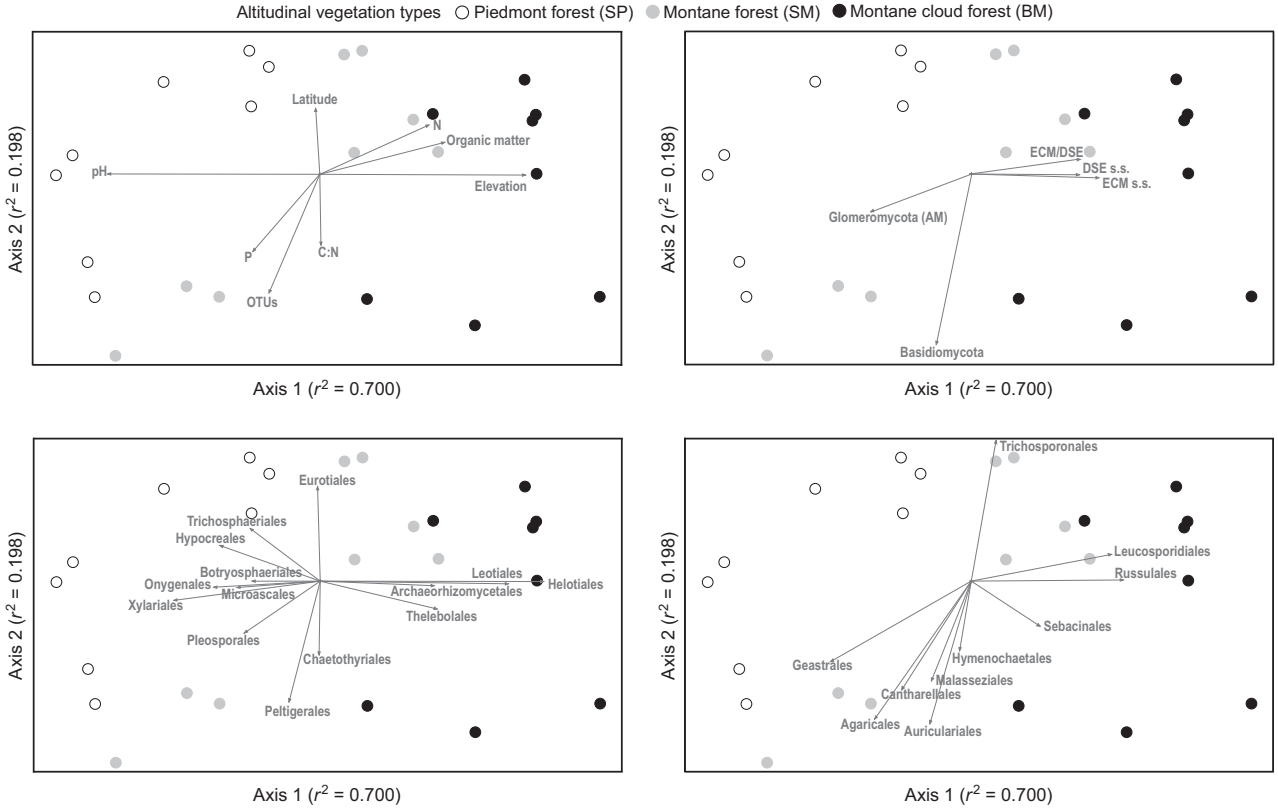


Fig. 4 Nonmetric multidimensional scaling (NMDS) ordination plots for fungal communities from various forest types. Labels, localities and descriptions of the sampling sites are given in Table 1. Because of the high number of variables tested, vectors of variables correlated with ordination axes at $|r| \geq 0.5$ are distributed over four identical ordination plots with abbreviations explained in Table 2.

insect pathogens (*Cordyceps* sp., *C. chlamydosporia*, *Metarhizium anisopliae*), plant pathogens (e.g. various *Fusarium* species, *Mycosphaerella areola*, *Bionectria rossmaniae*, *Cadonectria cerciana*) and saprobic fungi (*Cercophora coprophila*, *Ganoderma subamboinense*, *Paecilomyces marquandii*, etc.). The list of indicator OTUs is shown in Table 3.

Discussion

Large-scale fungal community patterns

Contrary to what has been observed for plants, where the piedmont and particularly the montane forests have been shown to harbour many more species than the montane cloud forests (Morales *et al*. 1995), we did not find substantial differences in soil fungal diversity among the three altitudinal zones. Our data suggest that total fungal diversity does not decrease significantly with increasing altitude among our sampling sites that ranged from approximately 400 to 2160 m a.s.l. Rather, different functional groups (which may or may not be apparent at the level of taxonomic orders, depending on their functional diversity) appear to prefer certain altitudinal forest zones, and such turnover of taxa along an elevation gradient apparently does not affect substantially the richness in each zone. The medium beta diversity values within and the high value among the forest types confirm that there is strong community turnover among the altitudinal zones. In addition, we observed a weak negative correlation between latitude and OTU diversity. Similar trends of decreasing taxonomic diversity with increasing latitude have also been observed in various plants and animals inhabiting the Argentinian Yungas, for example trees (Morales *et al*. 1995), mammals, and birds (Brown *et al*. 2001).

Despite the comparable fungal diversity measures, all statistical analyses suggest that fungal communities are strongly structured according to altitudinal forest types. Even though some OTUs were distributed across all or most sites, the majority of OTUs were restricted to certain vegetation types. Furthermore, the NMDS analyses suggest that fungal communities in the montane forests may be regarded as intermediate or transitional between communities of the piedmont and montane cloud forests. The Venn diagrams as well as the very

Table 3 OTUs considered as significant indicators of the altitudinal vegetation zones with corresponding *P*-values, and with accession numbers, sequence similarity, pairwise alignment length, name and taxonomic classification of the most similar sequence in the UNITE+INSD database. Only OTUs with at least 97% ITS2 sequence similarity to a closely related sequence are shown. When multiple OTUs matched the same sequence and were indicators of the same forest type, only the OTU with the highest sequence similarity to the reference sequence is shown. Where available, the species hypothesis (SH) numbers are given for the corresponding sequence as published by Kõljalg *et al*. (2013)

OTU	Zone	*P*	Accession	%	bp	Name	SH	Phylum	Order
1217	BM	0.0014	UDB002986	100.0	211	*Alnicola* sp.	SH139888.05FU	Basidiomycota	Agaricales
6879	BM	0.0224	AY900044	97.7	173	*Alnicola subconspersa*	—	Basidiomycota	Agaricales
1590	SP	0.02	EU816396	100.0	220	Ascomycota sp.	—	Ascomycota	—
1714	SP	0.0208	HQ607854	99.5	194	Ascomycota sp.	—	Ascomycota	—
3135	SP	0.004	AF210665	98.6	295	*Bionectria rossmaniae*	SH106284.05FU	Ascomycota	Hypocreales
1441	BM	0.015	HQ207061	99.7	286	*Botryosphaeriales* sp.	SH109008.05FU	Ascomycota	Botryosphaeriales
2264	SP	0.0242	GQ280559	99.3	276	*Calonectria cerciana*	SH111271.05FU	Ascomycota	Hypocreales
6839	BM	0.022	KC412006	98.9	188	*Calycina claroflava*	—	Ascomycota	Helotiales
2476	SP	0.0172	AY999136	98.6	286	*Cercophora coprophila*	SH113072.05FU	Ascomycota	Sordariales
1609	BM	0.0028	GU183124	97.1	280	*Chloridium virescens*	—	Ascomycota	Chaetosphaeriales
6375	BM	0.0074	EU624335	98.5	264	*Cladophialophora* sp.	SH108044.05FU	Ascomycota	Chaetothyriales
2388	SP	0.0048	KC176287	97.0	332	*Clitopilus* sp.	—	Basidiomycota	Agaricales
13772	SP	0.0196	AB100362	98.4	306	*Cordyceps chlamydosporia*	SH110361.05FU	Ascomycota	Hypocreales
1474	SP	0.0202	KC007316	99.5	203	*Cordyceps* sp.	—	Ascomycota	Hypocreales
7179	BM	0.0226	AY669684	97.0	298	*Cortinarius helvelloides*	—	Basidiomycota	Agaricales
4811	BM	0.0206	UDB014074	99.0	300	*Cortinarius* sp.	—	Basidiomycota	Agaricales
750	BM	0.0394	KC455901	100.0	196	*Cryptococcus fuscescens*	—	Basidiomycota	Filobasidiales
10944	BM	0.0078	FN298665	98.8	241	*Cryptococcus podzolicus*	—	Basidiomycota	Tremellales
1658	SP	0.0208	AF444385	99.6	273	*Cryptococcus* sp.	SH100400.05FU	Basidiomycota	Tremellales
588	SP	0.0222	FJ153168	99.0	305	*Cryptococcus* sp.	SH106532.05FU	Basidiomycota	Tremellales
2362	BM	0.011	FN298663	100.0	183	*Cryptococcus terricola*	—	Basidiomycota	Filobasidiales
2707	BM	0.018	JF519543	98.2	274	*Dothideomycetes* sp.	SH115786.05FU	Ascomycota	—
2328	BM	0.001	JX043059	98.1	259	ectomycorrhizal Helotiales	—	—	—
24735	SP	0.02	EF505350	97.8	267	endophytic fungus	SH102639.05FU	—	—
1432	SP	0.0028	HQ607852	100.0	309	*Exophiala* sp.	—	Ascomycota	Chaetothyriales
14396	BM	0.0422	FM866395	99.7	289	Filobasidiaceae sp.	SH117279.05FU	Basidiomycota	Filobasidiales
175	SP	0.0398	KC691556	100.0	172	*Fusarium ambrosium*	—	Ascomycota	Hypocreales
1604	SP	0.0328	AB820721	99.6	283	*Fusarium equiseti*	—	Ascomycota	Hypocreales
1738	SP	0.0136	KC429789	99.2	254	*Fusarium oxysporum*	—	Ascomycota	Hypocreales
2232	SP	0.0228	AB587014	99.0	197	*Fusarium phaseoli*	SH102222.05FU	Ascomycota	Hypocreales
3019	SP	0.0466	FN868470	100.0	269	*Fusarium proliferatum*	SH104185.05FU	Ascomycota	Hypocreales
3266	SP	0.0216	AB369415	97.9	188	*Fusarium solani*	—	Ascomycota	Hypocreales
19526	SP	0.0008	KF060154	97.2	211	*Fusarium solani*	—	Ascomycota	Hypocreales
7008	SM	0.0136	HQ731631	98.2	277	*Fusarium* sp.	SH102234.05FU	Ascomycota	Hypocreales
1924	SP	0.0146	JQ771178	98.1	212	*Fusarium* sp.	—	Ascomycota	Hypocreales
14830	SP	0.0212	AB369482	97.5	283	*Fusarium* sp.	—	Ascomycota	Hypocreales
108	SP	0.024	JQ520205	100.0	197	*Ganoderma subamboinense*	SH101846.05FU	Basidiomycota	Polyporales
1690	BM	0.0006	GQ996162	99.3	278	Helotiales sp.	SH117392.05FU	Ascomycota	Helotiales
1971	BM	0.011	HQ211548	99.3	280	Helotiales sp.	SH115846.05FU	Ascomycota	Helotiales
12914	BM	0.0046	HQ207068	97.7	214	Helotiales sp.	SH108407.05FU	Ascomycota	Helotiales
7277	BM	0.001	HQ211548	97.3	255	Helotiales sp.	SH115846.05FU	Ascomycota	Helotiales
26820	BM	0.0204	JX317195	97.2	281	Helotiales sp.	SH099548.05FU	Ascomycota	Helotiales
447	BM	0.0024	HM439557	99.7	298	Hypocreales sp.	—	Ascomycota	Hypocreales
14201	BM	0.0234	AM419065	98.0	297	*Ilyonectria radicicola*	—	Ascomycota	Hypocreales
14769	BM	0.0124	GQ922913	97.8	277	*Ilyonectria radicicola*	—	Ascomycota	Hypocreales
422	BM	0.0058	HQ604813	98.2	281	*Inocybe jacobi*	SH104866.05FU	Basidiomycota	Agaricales
16231	BM	0.0246	FN550883	97.3	183	*Inocybe jacobi*	SH104866.05FU	Basidiomycota	Agaricales
1659	BM	0.005	UDB014077	99.7	301	*Inocybe* sp.	—	Basidiomycota	Agaricales
1600	BM	0.0058	UDB002984	100.0	280	*Lactarius* sp.	SH100131.05FU	Basidiomycota	Russulales
1864	BM	0.0058	UDB014057	99.7	323	*Lactarius* sp.	—	Basidiomycota	Russulales
1482	SP	0.019	JX464062	99.7	291	*Lasiodiplodia theobromae*	—	Ascomycota	Botryosphaeriales
1578	BM	0.0054	AY706320	97.6	251	*Leohumicola verrucosa*	—	Ascomycota	Helotiales

Table 3 *Continued*

OTU	Zone	*P*	Accession	%	bp	Name	SH	Phylum	Order
746	BM	0.0004	HM230869	98.9	282	*Leotiomycetes* sp.	SH115850.05FU	Ascomycota	—
15260	SP	0.0042	JF792884	97.6	289	*Metarhizium anisopliae*	SH107811.05FU	Ascomycota	Hypocreales
6859	SP	0.0212	DQ767592	98.6	209	*Monascus purpureus*	SH104441.05FU	Ascomycota	incertae sedis
2501	SP	0.0188	AJ271630	99.5	192	*Mortierella alpina*	—	Zygomycota	Mortierellales
8698	SP	0.0046	JX976041	98.2	331	*Mortierella ambigua*	—	Zygomycota	Mortierellales
1674	BM	0.0054	FJ456972	100.0	237	*Mortierella* sp.	SH104362.05FU	Zygomycota	Mortierellales
2843	BM	0.005	JN104514	98.9	175	*Mortierella* sp.	—	Zygomycota	Mortierellales
14121	BM	0.004	AF504843	98.2	327	*Mortierella* sp.	SH117207.05FU	Zygomycota	Mortierellales
87	SP	0.0402	GU327522	97.3	259	*Mortierella* sp.	SH114748.05FU	Zygomycota	Mortierellales
27167	BM	0.0038	AB476419	98.8	255	*Mortierella verticillata*	—	Zygomycota	Mortierellales
26855	BM	0.0264	JX243801	97.0	265	Mortierellaceae sp.	—	Zygomycota	Mortierellales
1648	SP	0.0046	DQ459079	97.8	276	*Mycosphaerella areola*	SH104194.05FU	Ascomycota	Capnodiales
1597	SP	0.0368	EU925387	98.2	282	*Myriodontium keratinophilum*	—	Ascomycota	incertae sedis
443	SP	0.0208	JQ692125	97.3	300	Nectriaceae sp.	SH107227.05FU	Ascomycota	Hypocreales
1973	SP	0.0002	KC288117	99.6	285	*Nigrograna mackinnonii*	—	Ascomycota	Pleosporales
210	SP	0.0142	EU272503	98.6	288	*Nigrospora oryzae*	—	Ascomycota	Trichosphaeriales
2389	SP	0.0022	HQ631070	99.3	287	*Nigrospora* sp.	SH110463.05FU	Ascomycota	Trichosphaeriales
23056	SM	0.0196	AJ608984	98.5	326	*Paecilomyces carneus*	SH112968.05FU	Ascomycota	Eurotiales
7346	SP	0.0006	FJ765026	100.0	256	*Paecilomyces marquandii*	SH101737.05FU	Ascomycota	Eurotiales
3846	SP	0.0008	JQ846086	99.7	301	*Paecilomyces marquandii*	SH101737.05FU	Ascomycota	Eurotiales
15273	BM	0.02	EU914140	98.2	283	*Penicillium verruculosum*	—	Ascomycota	Eurotiales
906	SP	0.0238	AY339323	97.1	273	*Pilidiella diplodiella*	—	Ascomycota	Diaporthales
2106	BM	0.0224	EU035444	97.2	281	*Protoventuria alpina*	SH113984.05FU	Ascomycota	Pleosporales
213	SP	0.0194	GU053799	98.5	332	Psathyrellaceae sp.	SH108322.05FU	Basidiomycota	Agaricales
709	SP	0.0076	FJ765024	99.3	292	*Purpureocillium lilacinum*	SH114215.05FU	Ascomycota	Hypocreales
1653	SP	0.0368	NR_077089	98.1	258	*Rhodosporidium fluviale*	—	Basidiomycota	Sporidiobolales
2363	SP	0.0046	JX244023	98.7	307	*Sordariomycetes* sp.	—	Ascomycota	—
14110	BM	0.0014	UDB002951	98.3	296	*Thelephora alnii*	SH113307.05FU	Basidiomycota	Thelephorales
2477	BM	0.0014	UDB002982	99.1	351	*Tomentella ellisii*	SH112628.05FU	Basidiomycota	Thelephorales
93	BM	0.0046	DQ195591	99.2	259	*Tomentella* sp.	SH103074	Basidiomycota	Thelephorales
6572	BM	0.0058	UDB014056	100.0	199	*Tomentella sublilacina*	—	Basidiomycota	Thelephorales
543	BM	0.006	UDB002979	100.0	305	*Tomentella testaceogilva*	—	Basidiomycota	Thelephorales
14056	BM	0.019	JF519257	97.8	317	Trechisporales sp.	SH114084.05FU	Basidiomycota	Trechisporales
15040	BM	0.0018	JQ666774	97.5	276	Tremellales sp.	—	Basidiomycota	Tremellales
1612	BM	0.007	KC180726	99.6	282	*Trichocladium opacum*	—	Ascomycota	Sordariales
1422	BM	0.0214	JQ040318	99.0	191	*Trichoderma asperellum*	—	Ascomycota	Hypocreales
1607	BM	0.0288	JN943744	99.7	296	*Trichosporon wieringae*	SH106236.05FU	Basidiomycota	Trichosporonales
23201	BM	0.0066	AY315667	98.3	293	*Trichosporon wieringae*	SH106236.05FU	Basidiomycota	Trichosporonales
2350	BM	0.04	DQ888724	100.0	308	*Umbelopsis ramanniana*	SH100505.05FU	Zygomycota	Mucorales
670	SP	0.0036	JQ081807	100.0	217	unidentified fungus	SH101552.05FU	—	—
1956	SP	0.0166	GQ921720	100.0	215	unidentified fungus	—	—	—
2371	SP	0.0124	GU306892	100.0	273	unidentified fungus	SH109538.05FU	—	—
1460	SP	0.0118	JQ693162	99.7	294	unidentified fungus	SH100682.05FU	—	—
7405	SP	0.0208	GU308287	99.0	194	unidentified fungus	—	—	—
2295	BM	0.0184	HQ445985	98.9	263	unidentified fungus	—	—	—
4090	SP	0.016	FN397149	98.9	263	unidentified fungus	—	—	—
2138	BM	0.0054	DQ421309	98.8	326	unidentified fungus	SH111145.05FU	—	—
539	BM	0.0242	AF504842	98.7	236	unidentified fungus	SH100460.05FU	—	—
814	BM	0.021	KC588795	98.6	294	unidentified fungus	—	—	—
1703	SP	0.001	GQ996096	98.6	288	unidentified fungus	—	—	—
2186	BM	0.0242	JX915590	98.4	249	unidentified fungus	—	—	—
134	SM	0.0212	GU315847	98.4	258	unidentified fungus	—	—	—
4856	SP	0.0494	JN578638	98.2	219	unidentified fungus	—	—	—
12788	BM	0.0246	GU366719	98.1	267	unidentified fungus	SH101063.05FU	—	—
120	SP	0.0048	GU309178	97.6	246	unidentified fungus	SH108787.05FU	—	—
2487	SP	0.0368	GU308287	97.6	250	unidentified fungus	—	—	—
2361	SP	0.0448	FJ386862	97.4	389	unidentified fungus	SH114701.05FU	—	—

Table 3 *Continued*

OTU	Zone	*P*	Accession	%	bp	Name	SH	Phylum	Order
2042	BM	0.0204	GU174340	97.3	334	unidentified fungus	—	—	—
5845	SP	0.0036	FJ386853	97.3	366	unidentified fungus	SH114741.05FU	—	—
2484	SM	0.003	EF121861	97.5	278	*Volutella consors*	—	Ascomycota	Hypocreales
2297	SP	0.0004	JQ693169	99.6	279	*Volutella lini*	SH100684.05FU	Ascomycota	Hypocreales
633	SP	0.0428	AY909016	97.9	287	*Xylaria longipes*	—	Ascomycota	Xylariales

Abbreviations for altitudinal vegetation zones are BM, montane cloud forest; SM, montane forest; SP, piedmont forest.

low number of indicator species in this mid-elevation zone suggest that many species found in the montane forests are also found in at least one other altitudinal zone. Our study documents a strong correlation between vegetation and fungal community composition in the Yungas, where vegetation can influence fungal communities, and *vice versa*, in a variety of direct and indirect ways, for example by providing hosts for symbiotic and substrate for decomposing fungi, and by altering environmental (microclimatic, edaphic, etc.) factors. There are presumably many fungal taxa that exhibit certain host or substrate specificity and, therefore, are restricted to sites where their plant, animal or fungal hosts occur.

The highest diversity of ECM fungi in our data set was found in montane cloud forest sites dominated by *Alnus acuminata* (e.g. sites BM2, BM3, BM4, BM5, BM7 and BM8). The vast majority of these OTUs were highly similar to known alder symbiotic ECM fungi (Table 3). Therefore, our results confirm former reports of strikingly high sequence similarity among various alder-associated ECM fungi across distant geographic areas (Becerra *et al.* 2002, 2005a; Tedersoo *et al.* 2009b; Pritsch *et al.* 2010; Kennedy *et al.* 2011) and, thus, provide further support to the hypothesis of recent co-migration of these ECM fungi with *Alnus* from the Northern Hemisphere as discussed by Kennedy *et al.* (2011). Interestingly, there were also several unidentified Thelephoraceae OTUs only found in the subtropical montane forest and piedmont zones that were related to species sequenced from Neotropical Nyctaginaceae hosts. While all alder-associated ECM fungal sequences had high similarity values to reference sequences as mentioned previously, the ECM fungi in the lower elevations had generally low similarity values to the most similar publicly available sequences (ranging from 91.23% to 94.40%). Therefore, these subtropical forests apparently harbour at least one or more divergent lineages of thelephoroid fungi that have not been sequenced previously and that may be associated with *Pisonia* species that are known to occur in the montane and piedmont forests (E. Nouhra pers. obs.). Future studies should include ECM root tip samples from Nyctaginaceae species to learn more about the diversity and host preference of these fungi. Finally, we also found sequences of various *Sebacina* and related genera in multiple sites in all three altitudinal zones. However, given the taxonomic difficulties and the wide spectrum of ecological roles in the Sebacinales (Weiss *et al.* 2011), we do not know whether these represent ECM, ericoid mycorrhizal, root endophytic or orchid mycorrhizal lineages. Future projects based on analyses of root samples should clarify the functional roles of these taxa.

The high diversity and heterogeneity of plant and animal hosts, and the similarly varied decomposing plant substrates in the Yungas presumably support diverse communities of pathogenic, leaf endophytic and saprobic fungi. Given that the diversity of plants and animals is considered substantially higher in the warmer, lower altitudinal zones than in the more temperate montane cloud forest (Brown *et al.* 2001), taxonomic groups containing numerous endophytic, wood-decaying and plant or animal pathogenic species are expected to be more diverse at lower elevations. Indeed, the order Hypocreales, rich in insect- and plant pathogens as well as in endophytes and wood decayers, appears to be most diverse in the piedmont zone, and it also included the majority of the indicator species for that zone. Similarly, the observed numbers of OTUs in the Xylariales, which also includes many leaf endophytes and wood-decay fungi, and in Onygenales, comprising many keratin-degrading species primarily found on animals and dung, were highest in the piedmont forests.

Environmental factors shaping fungal community composition

Although environmental factors influencing the distribution of plants and animals have been thoroughly studied, environmental factors controlling the spatial patterns and community composition of soil microorganisms are still poorly understood (Rousk *et al.* 2010). Our data show that fungal community structure in the Yungas is primarily shaped by altitude, presumably because, besides temperature, it greatly influences the amount of precipitation and relative humidity in

this orographic region. Studies on fungal communities along altitudinal gradients are very scarce and are often specimen-based and limited to certain taxonomic groups. Former studies based on AM fungi and cultured microfungi in the Himalayas (Devi *et al.* 2012; Gai *et al.* 2012), ECM root tips in Iran (Bahram *et al.* 2012), fruitbodies of hypogeous ECM fungi in Patagonia (Nouhra *et al.* 2012), and fruitbody surveys of ECM and saprobic macrofungi in Mexico (Gómez-Hernández *et al.* 2012) also found significant effects of elevation on species richness, although the distributional patterns were different. For example, Devi *et al.* (2012) observed that the diversity of culturable microfungi (mainly ascomycetes in Hypocreales and Eurotiales) showed negative correlation with altitude and soil moisture content and positive correlation with soil temperature and soil pH. This is in agreement with our observations where values of OTU richness in various groups of ascomycetes, including Hypocreales and Eurotiales, were greater at the drier, warmer and more alkaline piedmont forest sites. Similarly, Gai *et al.* (2012) found that richness of AM fungi showed a decreasing trend with increasing elevation that corresponds well to our observations that glomeromycetes are more diverse in the low altitudinal zones of the Yungas. Bahram *et al.* (2012) and Nouhra *et al.* (2012) found declining richness with increasing altitude in ECM fungi in temperate mountain forests in northern Iran and in Patagonia, respectively, while models for macromycete richness (including ECM fungi) in Mexico have been reported to peak in mid-elevation cloud forests (Gómez-Hernández *et al.* 2012). Although we also found the highest diversity of ECM fungi in the cloud forest zone, it is difficult to compare our results with these previous works because of the scarcity of ECM host tree families in the lower elevation zones in our sampling region. This underlies the general importance of host plant family in shaping the community composition of ECM fungi, as discussed by Tedersoo *et al.* (2012). Besides the edaphic factors, the turnover of decomposing organic matter is known to be faster at higher temperatures, that is, at lower elevations (Rastin *et al.* 1990; Osono 2007), and this may positively influence the diversity of pathogenic and decomposing fungi that are well represented in ascomycetes in general, and in Hypocreales, Onygenales and Eurotiales in particular (Alexopoulos *et al.* 1996; Kirk *et al.* 2008; Houbraken *et al.* 2012). Similar patterns have been observed in altitudinal gradients in both temperate and tropical areas established from fruitbody-based or culturing studies, that is, diversity and abundance of decomposer fungi are generally greater at lower altitudes (Rastin *et al.* 1990; Osono 2007; Gómez-Hernández *et al.* 2012). This is in agreement with our observations, as discussed above.

Soil pH is thought to play an important role in shaping fungal communities with supporting evidence provided by both experimental studies and sequencing studies of fungal communities (Porter *et al.* 1987; Coughlan *et al.* 2000; Lauber *et al.* 2008; Rousk *et al.* 2010). Our large-scale comparison of sites with various acidity levels indicates a strong correlation between soil pH and the composition of fungal communities in the Yungas (Fig. 4, Table 2). Because many fungal species have a relatively wide pH optimum (Wheeler *et al.* 1991; Nevarez *et al.* 2009), it is likely that the observed correlation of pH with community composition is mainly indirect, for example via nutrient availability and altered competitive interactions between soil fungi and bacteria (Rousk *et al.* 2010), and other soil biota. Alternatively, because soil pH had a strong negative correlation with elevation in our samples, its correlation with fungal community composition may not be causal. As our study focuses on natural systems, disentangling such causal relationships is beyond the scope of our work. Nevertheless, for some fungal groups that are known to be influenced by soil pH, our data are in agreement with previous results. For example, AM fungi had been found to prefer relatively high soil pH (Porter *et al.* 1987; Coughlan *et al.* 2000), and in our data set, they were indeed more diverse in sites with higher pH values, with a correlation of $r = 0.5820$. DSE fungi had been shown to prefer low soil pH, and more intensive DSE colonization had been observed in acidic conditions in European beech forest sites possibly due to their high H^+ tolerance (Postma *et al.* 2006) and/or higher competition pressure from other fungi at neutral or alkaline pH levels (Porter *et al.* 1987; Bartolome-Esteban & Schneck 1994; Postma *et al.* 2006). Similarly, ECM fungi are generally considered acidophilus (Read 1991), and previous studies of ECM fungi from the Yungas suggested that they are adapted to acidic soils (Becerra *et al.* 2005a). In our data, we found the highest diversity of both DSE and ECM fungi in the low pH sites in the montane cloud forest.

Overall, it seems likely that the orographic characteristics of the sampled sites determine the key abiotic factors (temperature and precipitation) that, in turn, result in complex interactions between biota and edaphic factors. For example, at higher elevations, the higher precipitation and the greater accumulation of litter due to lower decomposition rates at these lower temperatures (as also indicated by greater soil organic matter content) influence soil pH and the biotic communities. Our data showed that, besides elevation and soil pH, the composition of fungal communities was also correlated with organic matter content, N content and C/N ratio in the soil. Highest organic matter and N contents are characteristic of montane cloud forests, particularly

those formed by *Alnus acuminata* that, like other members of the genus, is able to fix atmospheric N via bacterial nodules (Becerra *et al.* 2005a,b, 2009b). In addition, the greater diversity and abundance of ECM fungi in *Alnus* forests probably contribute to the accumulation of soil organic matter through the continuous addition of fixed C to the rhizosphere. This mechanism of soil C accumulation derived from roots and root-associated fungi has been shown to contribute 50–70% of stored C in ECM-dominated boreal forests (Clemmensen *et al.* 2013).

Conclusions

Large-scale DNA sequencing projects have immense potential to augment our current knowledge of fungal diversity and to better understand environmental factors that influence fungal community structure. Such undertakings are particularly important in poorly sampled and presumably highly diverse tropical and subtropical areas, such as the region of our study. Despite the presence of more than 350 000 fungal ITS sequences in public databases (Kõljalg *et al.* 2013), more than half of the OTUs in our samples could not be identified to species or species complexes. Because of the rigorous quality checks applied while processing the sequence data, our conservative estimates clearly indicate the need for further mycological research in the Yungas in particular and in the Neotropics in general. Many fungi at the sampling sites probably are still undescribed, while others may remain unidentified because of the unavailability of reference sequence data from known species. Building a near-exhaustive reference sequence library for already described species with available voucher specimens, for example via large-scale DNA barcoding efforts at herbaria and culture collections, is urgently needed to close the gap in our knowledge and to make better use of high-throughput soil sequencing projects.

Our work provides an unprecedented insight into the diversity of fungal communities in the Yungas. Although more detailed, taxon-specific studies will follow, this work offers examples for the potential contribution of high-throughput soil sequencing studies to biodiversity assessments and landscape ecology that can provide crucial field data for conservation efforts.

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J.G., A.B. and E.N. conceived the research idea, and J.G., E.N., L.F. and N.P. carried out the soil sampling for this paper. T.S. extracted the DNA, carried out PCR and prepared the samples for the Ion Torrent run. S.P. provided maps and site information as part of the preparation for the fieldwork and prepared the maps for Fig. 1. J.G. conducted the bioinformatic and statistical analyses and wrote the first draft of the manuscript. C.W. provided help with database manipulation of the BLAST results and the site vs. OTU matrices. All authors provided comments on the manuscript.

Data accessibility

The raw sequence data, the representative sequences of OTUs, and the OTU distribution matrix have been submitted to Dryad: doi:10.5061/dryad.8fn8j.

Supporting information

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

Table S1 Ion Torrent adaptors, primers, and Multiplex Identification DNA-tags (MIDs) used in this study.

Table S2 The top 200 OTUs by sequence count and the corresponding most similar publicly available sequences.

Table S3 Pairwise correlation values, *r*, of environmental variables of the sampled Andean montane forest sites.