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Host associations and beta diversity of fungal endophyte communities in New Guinea rainforest trees

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Abstract

Processes shaping the distribution of foliar fungal endophyte species remain poorly understood. Despite increasing evidence that these cryptic fungal symbionts of plants mediate interactions with pathogens and herbivores, there remain basic questions regarding the extent to which dispersal limitation and host specificity might shape fungal endophyte community composition in rainforests. To assess the relative importance of spatial pattern and host specificity, we isolated fungi from a sample of mapped trees in lowland Papua New Guinea. Sequences of the internal transcribed spacer (ITS) region were obtained for 2079 fungal endophytes from three sites and clustered into molecular operational taxonomic units (MOTUs) at 95% similarity. Multivariate analyses suggest that host affinity plays a significant role in structuring endophyte community composition whereas there was no evidence of endophyte spatial pattern at the scale of tens to hundreds of metres. Differences in endophyte communities between sampled trees were weakly correlated with variation in foliar traits but not with tree species relatedness. The dominance of relatively few generalist endophytes and the presence of a large number of rare MOTUs was a consistent observation at three sites separated by hundreds of kilometres and regional turnover was low. Host specificity appears to play a relatively weak but more important role than dispersal limitation in shaping the distribution of fungal endophyte communities in New Guinea forests. Our results suggest that in the absence of strong ecological gradients and host turnover, beta diversity of endophyte communities could be low in large areas of contiguous forest.

Keywords: angiosperms, community ecology, DNA barcoding, fungi, species interactions

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Introduction

Symbiotic relationships between fungi and plants span deep evolutionary history, from the colonization of land by plants (Simon *et al.* 1993) to contemporary facultative and obligate interactions between fungi and plant roots, stems, and foliar tissue. A growing body of research has shown that fungal symbionts may strongly influence plant function and plant–herbivore interactions in ways that could shape distributions of biodiversity at large scales (Peay *et al.* 2013). In particular, foliar fungal endosymbionts have been shown to mitigate pathogen damage (Arnold 2003; Busby *et al.* 2013) and influence plant–insect interactions (Breen 1994; Omacini *et al.* 2001). Despite the apparent ecological importance of fungal endophytes, we have only a nascent understanding of fundamental processes that structure endophyte communities in forest systems (U'Ren *et al.* 2012; Zimmerman & Vitousek 2012). In this work, we evaluate two hypotheses for ecological processes that could influence the distribution of diversity in foliar endophyte communities, namely host associations and dispersal limitation.

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Foliar fungal endophytes are ubiquitous to photosynthetic land plants and lichens, occupying healthy tissues without causing outward signs of infection (Petrini 1991; Arnold & Lutzoni 2007). Most fungal endophyte taxa are horizontally transmitted among diverse photobiont taxa with colonies occupying small patches within the plant tissues (Arnold & Lutzoni 2007) while the systemic endophytes of temperate grass species (Saikkonen *et al.* 1998; Schardl *et al.* 2004) are often vertically transmitted and involve a limited group of taxa in the Clavicipitaceae (Rodriguez *et al.* 2009). Interactions between the host plant and fungal endosymbionts span the spectrum from mutualism to commensalism to parasitism (Faeth & Sullivan 2003) although they are often considered to be beneficial to their hosts (Carroll 1988; Wilson 1995; Saikkonen *et al.* 1998; Rudgers *et al.* 2012).

Host associations and dispersal limitation are hypothesized to play fundamental roles in structuring fungal endophyte communities. Historically, the Baas-Becking hypothesis, 'everything is everywhere, the environment selects', predicts that microbial species disperse widely and that survival to reproduction is determined by the local environment including associations with particular host species. The species composition of an endophyte community that is shaped by host association could be correlated with host distribution as well as abiotic conditions preferred by the host. Depending on the degree of specificity in host associations, correlations might be found at differing host taxonomic levels or with phylogenetically conserved traits of plant taxa. Alternatively, dispersal limitation theory posits that the probability of an endophyte colonizing a new location is inversely related to the spatial distance between locations. Dispersal is a fundamental process that structures biotic communities in space and time (e.g. Nekola & White 1999; Soininen *et al.* 2007). If endophyte species are primarily limited by their dispersal ability, we predict that adjacent communities will be more similar than are more distant communities. Both host associations and dispersal limitation predict species turnover with distance. However, whereas the host association hypothesis implies that the distribution of individual microbial taxa depends on their evolved host associations and thus predicts correlation with the spatial structure of host individuals and populations, the dispersal limitation hypothesis implies a stochastic process by which communities are assembled from the regional species pool (Hubbell 2001; Condit *et al.* 2002). We used the contiguous extent of rainforests in New Guinea to examine the relative importance of host association, dispersal limitation and their interaction in structuring fungal endophyte communities.

Factors affecting foliar fungal endophyte community composition have been examined primarily in the temperate zone (e.g. U'Ren *et al.* 2012) and to a lesser extent in the tropics. U'Ren *et al.* (2012) showed strong geographic structure driven by both abiotic variables and host associations at a continental scale in temperate and boreal forest fungal endophyte communities. In the tropics, elevational gradients (Zimmerman & Vitousek 2012) and variation in soils (Higgins *et al.* 2014) may strongly influence endophyte community structure. To our knowledge, no study has investigated host associations of endophytic fungi across a phylogenetically diverse set of rainforest trees and turnover of community diversity in the absence of strong ecological gradients.

Variation in endophyte community composition emerging from the interplay of host specificity and dispersal limitation could have significant implications for fungal community ecology. For example, the presence of fungal endophytes has been shown to decrease foliar pathogen damage in *Theobroma cacao*, an economically important tropical tree (Arnold 2003). Such defensive mutualisms could lead to a competitive advantage to hosts (e.g. Clay & Holah 1999; Booth 2004) in the presence of pathogens or herbivores and might thereby influence the structure of rainforest tree communities. Regardless of the nature of these symbioses, foliar fungal endophytes represent a cryptic reservoir of biodiversity. Understanding factors shaping the distribution of these fungi could also aid bioprospecting efforts that aim to discover diverse secondary metabolites and novel bioactive compounds (Higginbotham *et al.* 2013; Ortega *et al.* 2013).

With the goal of understanding distributional patterns of foliar fungal endophytes, we examined five questions about the endophyte communities of New Guinea rainforest trees.

1 To what extent do fungal endophyte communities differ among host tree species?
2 Are differences in endophyte community similarity explained by phylogenetic distance among hosts?
3 Are patterns of endophyte distribution associated with chemical and structural features of foliage?
4 Is endophyte community composition related to the spatial distance between host trees sampled at a given location?
5 What fraction of a local endophyte community is shared among trees at a regional scale?

Materials and methods

To address these questions, we sampled foliage in lowland rainforests of Papua New Guinea, including the fully mapped 50-ha forest dynamics plot at Wanang (Vincent *et al.* 2015). Fungal endophytes were sampled from a phylogenetically diverse group of rainforest tree

species following standard protocols for isolation and culture (Arnold *et al.* 2001, 2007). Sampled endophytes were sequenced for the internal transcribed spacer region (ITS) of ribosomal DNA and grouped into 95% similar molecular operational taxonomic units (MOTUs). Data were analysed in a multivariate framework to take advantage of our spatially explicit, highly dimensional data and to address specific questions on factors affecting patterns of local variation and regional turnover in fungal endophyte community composition.

Study sites

The island of New Guinea is one of the world's largest tropical forest areas (Mittermeier *et al.* 1998) with 33 million hectares on the eastern half of the island in the nation of Papua New Guinea (Shearman *et al.* 2008; Shearman & Bryan 2011). Approximately 19 million hectares (Shearman *et al.* 2008; Shearman & Bryan 2011) of this forest area is lowland rainforest. Although threatened by deforestation (Shearman *et al.* 2008), these ever wet lowland rainforests are reasonably intact and constitute an essentially contiguous forest landscape.

Endophytes were sampled in mature lowland wet rainforest in the Madang and East Sepik provinces of northern Papua New Guinea during 2010 and 2011 (Fig. 1A). In 2010, endophytes were sampled exclusively in the Wanang forest dynamics plot (FDP), and in 2011, samples were obtained at the Wanang FDP and, Ohu and Wamangu sites. The Wanang FDP was established using the protocol of the Smithsonian Center for Tropical Forest Science (CTFS), a global network of forest plots (Condit 1995). As in other plots across the network, all trees larger than 1 cm in diameter were measured, tagged, mapped and identified to species. Detailed site descriptions of Ohu, Wamangu and the Wanang FDP can be found in Novotny *et al.* (2007), Craft *et al.* (2010) and Vincent *et al.* (2015), respectively.

Host species sampled

Host species were chosen to encompass phylogenetic breadth among the rainforest trees of lowland New Guinea (Fig. 1B). In both years, three species of *Ficus* (Moraceae) and three species of *Macaranga* (Euphorbiaceae) were sampled. These large and widespread genera were sampled to examine differences between endophyte communities associated with closely related host species and were compared with communities hosted by more distantly related genera including *Psychotria* (Rubiaceae), *Syzygium* (Myrtaceae), and *Gnetum*, a broadleaved gymnosperm. Species distribution maps of all sampled species in the Wanang 50-ha forest dynamics plot are provided in Fig. 2. At least nine host tree species were sampled in 2010 and at each of the three sites in 2011 but with minor differences in host trees sampled among sites (Table S1). For example,

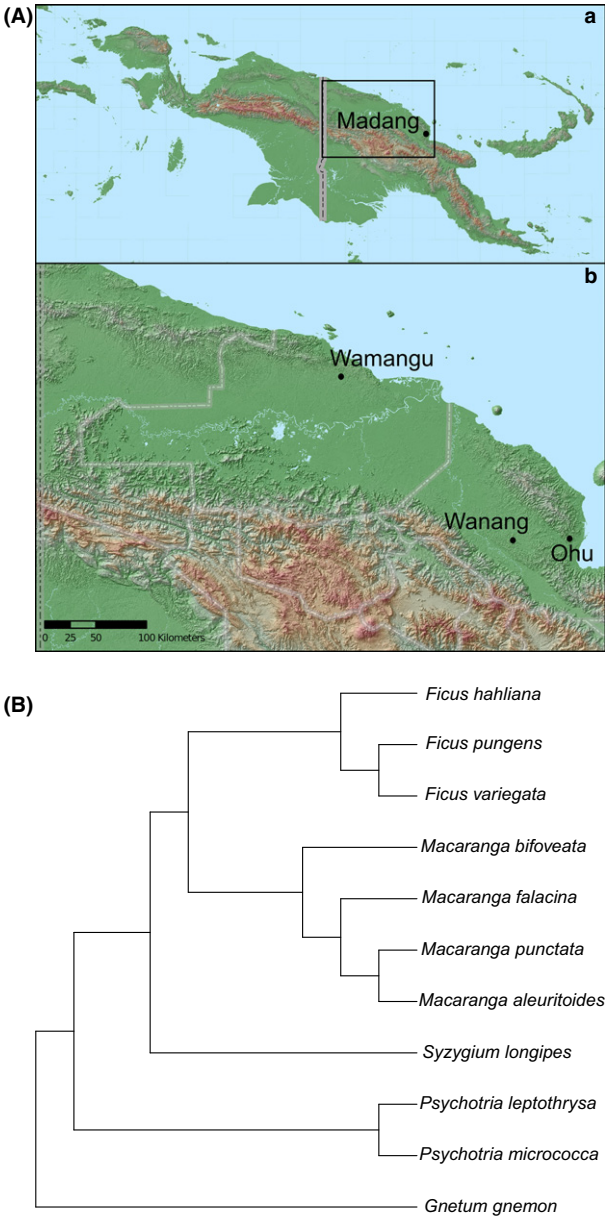

Fig. 1 Sampling locations in the lowlands of northern Papua New Guinea in the Madang and East Sepik Provinces (A). Sites are located in contiguous lowland rainforest in the Ramu (Ohu and Wanang) and Sepik (Wamangu) river valleys. Vegetation, climate and elevation are very similar at all sites. A diverse set of rainforest trees was sampled at each site (B). Multiple species from widespread and abundant genera (*Ficus* and *Macaranga*) as well as the broadleaved gymnosperm, *Gnetum gnemon*, were sampled. The dendrogram shown includes all sampled host species and reflects the phylogenetic hypothesis used in this study. Dendrogram branch lengths do not correspond to a measure of relatedness.

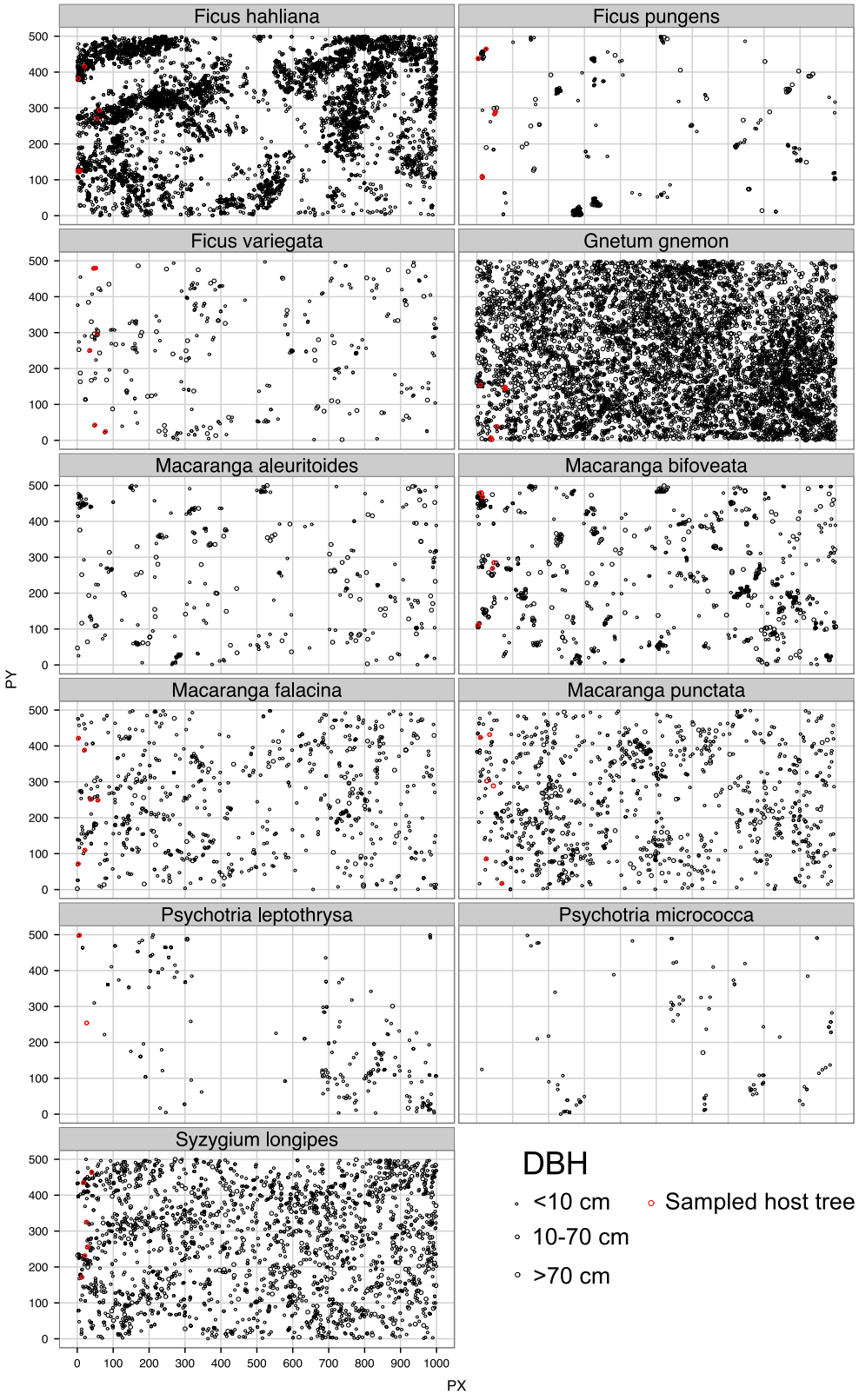

Fig. 2 Spatial distributions of all sampled host trees in the Wanang 50-ha forest dynamics plot. Each point corresponds to an individual tree with trunk diameter represented by the size of the point. Axes are scaled in metres corresponding to plot dimensions of 500 m by 1000 m. Locations of sampled host trees are shown in red and are found in a linear swath on the left end of plot maps.

sampling in 2010 at Wanang included *Gnetum* but not *Psychotria micrococca*. In 2011, sampling at Ohu included *Macaranga aleuritoides* in place of *Macaranga falacina* and at Wamangu, *Psychotria leptothyrsa* could not be located. Previous work on rainforest trees at Wanang (Whitfeld *et al.* 2012b, 2014) provided measurements of leaf traits (specific leaf area, leaf nitrogen and leaf carbon) for each of the host species sampled at the Wanang site. Sampling of trees at Ohu and Wamangu in 2011 was opportunistic and not spatially explicit.

Leaf and endophyte sampling

All samples were collected from lamina of fully expanded sun leaves showing no signs of damage or disease. In 2010, six fully expanded full sun leaves were collected from each host tree. Six trees per species were sampled, except in the case of *Psychotria leptothrysa* for which only two trees could be located. Leaves were returned to a field laboratory where they were surface sterilized using successive washes in solutions of ethanol and bleach (Arnold *et al.* 2001). Subsequently, four ~5-mm^2 square segments were excised from each leaf. Each leaf segment was plated on 2% malt extract agar (MEA) media. Emergent fungal colonies were transferred to sterile culture tubes containing 2% MEA. In 2011, samples were collected from three trees per species per site. For each of six leaves per tree, ten fragments of approximately 2mm^2 were excised, surface sterilized and each placed directly into a 'mini-slant' of 2% MEA in an Eppendorf tube (Arnold *et al.* 2007). Tubes were exported by the University of Papua New Guinea and imported by University of Minnesota under APHIS permits P526P-07-06697 (2010) and P526P-11-02984 (2011) for culturing and DNA sequencing. Endophytes sample sets are referred to by site and year: Wanang 2010, Wanang 2011, Wamangu 2011 and Ohu 2011.

DNA extraction and amplification

Cultured fungal tissues were obtained either directly from colonies in mini-slants or from Petri dish cultures established from the stock mini-slants. Fungal tissue was disrupted using a Qiagen TissueLyser for 1 min at maximum RPM, and total genomic DNA was extracted using either Qiagen Plant Mini Kit (Qiagen) or Extract-N-Amp kits (Sigma-Aldrich).

After DNA extraction, polymerase chain reaction (PCR) was used to amplify the ITS region, a commonly used fungal barcoding region. Primers were selected to produce amplification of rDNA covering the ITS1 and ITS2 regions. Reactions were carried out in 20 μL volumes with 10 μM final concentrations of the forward ITS1-F and reverse ITS4 primers (White *et al.* 1990; Gardes & Bruns 1993) under the following conditions: initial denaturation 94 °C for 10 min, followed by 40 cycles of 94 °C for 45 sec, 55 °C for 45 s, 72 °C for 90 s, and final elongation 72 °C for 5 min.

Amplified fragments were Sanger sequenced using Big Dye sequencing reagents and protocols (Applied Biosystems, Foster City, CA, USA), and data were collected using an ABI Prism 3730XL DNA Analyser (Applied Biosystems). Sequences were processed for quality and manually trimmed using GENEIOUS 6.1.8 (Biomatters Limited). Basic local alignment search tool (BLAST) (Altschul *et al.* 1990) queries of the fungal User-friendly Nordic ITS Ectomycorrhizal Database (UNITE version 7) (Kõljalg *et al.* 2013). The UNITE database was originally designed to aid in the identification of ectomycorrhizal fungi (Kõljalg *et al.* 2005) but has since been extended to include over 300 000 high-quality ITS sequences from all major lineages of fungi including the Ascomycota. Sequences were deposited in GenBank under Accession numbers KR014946–KR017025.

Endophyte ITS sequences were clustered into MOTUs based on 95% ITS sequence similarity using the MOBYLE workflow (Monacell & Carbone 2014) that streamlines MOTHUR (Schloss *et al.* 2009) and ESPIRIT (Sun *et al.* 2009). Additional details on these clustering methods are presented in U'Ren *et al.* (2012) and have since been compiled into the workflow described in U'Ren *et al.* (2014). The 95% sequence similarity level for MOTUs corresponds approximately to the species level in endophyte phylogenies (Arnold *et al.* 2007) and allows comparison to relevant recent studies (U'Ren *et al.* 2012; Zimmerman & Vitousek 2012; Higgins *et al.* 2014). Sequences from 2010 and 2011 were clustered and analysed separately due to differences in sampling intensity, collection method and culturing.

Statistical analyses

All statistical analyses and data manipulations, except where explicitly stated otherwise, were carried out in R 3.2.2 (R Development Core Team 2015). We calculated descriptive summary statistics and produced visualizations of multivariate patterns in endophyte community composition for all sites. Species accumulation curves and standard deviations were constructed by repeated random addition of isolates without replacement. The distributions of endophyte MOTUs among host species and sampling sites were plotted as quantitative webs (Lewis *et al.* 2002) using the bipartite R package (Dormann *et al.* 2008). Variation in endophyte community composition among sampled trees was visualized by nonmetric multidimensional scaling (NMDS) using the function 'METAMDS' in the vegan R package (Oksanen

et al. 2013) on a Bray–Curtis dissimilarity matrix (Bray & Curtis 1957). The Bray–Curtis matrix was square root transformed to limit the influence of abundant MOTUs. Pairwise phylogenetic distance between sampled host species was estimated under maximum likelihood using previously published molecular data and a general time-reversible model accounting for rate heterogeneity and invariant sites (Silvieus *et al.* 2008; Novotny *et al.* 2010; Whitfeld *et al.* 2012a) using the APE package (Paradis *et al.* 2004).

Associations of endophyte community structure with host species, host phylogeny and leaf traits were analysed in a multivariate statistical framework. All analyses were carried out in parallel on data sets with and without singleton MOTUs included in order to assess the sensitivity of results to the inclusion of rare taxa. We performed simple Mantel tests for correlations between transformed Bray–Curtis matrices and pairwise host phylogenetic distance to examine the association between endophyte community composition and host tree relatedness. A null distribution drawn from 999 permutations of the Bray–Curtis matrix was used to test for statistical significance.

Differences in endophyte community species composition among host species and host genera were evaluated using permutational multivariate analysis of variance (PERMANOVA) (Anderson 2001), implemented as 'ADONIS' in the R VEGAN package (Oksanen *et al.* 2013). PERMANOVA determines the significance of variance in distance matrices associated with a priori groups such as host taxa. We evaluated Bray–Curtis endophyte community distance as a function of phylogenetic distance among species and genera by comparing the observed community matrix to a nonparametric null distribution based on 999 permutations of the observed matrix. Permutations evaluated the null hypothesis of exchangeability among groups. Given the potential susceptibility of PERMANOVA to elevated type I error in the case of heterogeneous variance among groups, we examined homogeneity of variance using a permutational test of multivariate group dispersions (PERMDISP) (Anderson 2006; Anderson *et al.* 2006), implemented as 'BETADISPER' in the VEGAN R package (Oksanen *et al.* 2013). Global and pairwise permutation tests were also performed to test for differences in dispersion (multivariate variance) between fungal communities associated with host species and genera using null distributions based on 999 permutations.

We evaluated correlations of fungal community diversity with leaf functional traits to investigate the potential for traits to influence the habitability of foliage. Three functional traits including specific leaf area (SLA), foliar nitrogen and foliar carbon previously characterized for New Guinea tree forest species (Whitfeld *et al.* 2012b) are known to correlate with plant function (Wright *et al.* 2004) and life history strategy (Wright *et al.* 2010). Specific leaf area (fresh leaf area/dry leaf mass, $cm^2\ g^{-1}$) reflects the allocation of resources to photosynthetic tissue vs. structural features (Agrawal & Fishbein 2006). Foliar nitrogen and carbon (as per cent leaf mass) are correlated with plant photosynthetic rate (Reich *et al.* 1994) and leaf toughness and longevity (Poorter *et al.* 2004), respectively. We explored correlations among these leaf traits and endophyte community composition using distance-based redundancy analysis (db-RDA) (Legendre & Anderson 1999; Anderson & Willis 2003). Ordination was performed on a square-root-transformed Bray–Curtis matrix of endophyte community composition with either all three traits or each trait individually. Statistical significance and the proportion of total variation in endophyte community composition explained by host traits were estimated using permutational ANOVA (199 permutations). Two *Psychotria* species were excluded from these analyses because trait data were not available.

We compared the relative explanatory power of tree location, species identity and functional traits using PERMANOVA. Variation in the distance matrix of endophyte community composition (Bray–Curtis) from host trees sampled in 2011 was partitioned with two models that loaded explanatory variables either in the order of location, species, and traits or location, traits and species. The two models were compared because variable order and the sequential sum of squares influence PERMANOVA results.

Spatial structure in endophyte community composition was investigated across local and regional scales for evidence of dispersal limitation. We tested for spatial structure in endophyte community composition at the scale of hundreds of m^2 using spatial coordinates to the nearest 10 cm for each sampled tree available in the Wanang FDP. Mantel tests as implemented in the vegan package (Legendre & Legendre 2012; Oksanen *et al.* 2013) evaluated structure of fungal endophyte community composition across all sampled host trees, conspecific hosts and congeneric hosts. For each test, the correlation was evaluated between a pairwise matrix of Euclidean distances among host trees and a square-root-transformed Bray–Curtis matrix of endophyte community composition.

We investigated beta diversity, a measure of differences in community composition (species turnover) between sites, using the Chao–Sorensen index (Chao *et al.* 2005; Anderson *et al.* 2010). This measure incorporates a probabilistic adjustment to account for species that might be present but unobserved due to undersampling of hyperdiverse communities (e.g. Novotny *et al.* 2007). The resulting index is the proportion of species shared between two sites, corrected for unobserved spe-

cies. Collections were rarefied to equalize sample sizes for the analyses of beta diversity between the three regional sites (Wanang, Ohu, Wamangu), and between host genera and species. Although this index has been shown to be robust to rarefaction and unequal sample sizes (Chao *et al.* 2005), we compared results from complete and rarefied data sets to explore how uneven sample sizes might affect estimates of beta diversity. We also investigated the sensitivity of our estimate of beta diversity to the exclusion of either the most abundant or the most rare taxa. To examine the effect of excluding abundant MOTUs, we analysed data sets in which either the most abundant MOTUs were excluded or in which the top five most abundant MOTUs were excluded. We examined the effect of excluding rare endophytes by analysing data sets excluding singletons or MOTUs with fewer than three observations (doubletons and singletons excluded), or MOTUs with fewer than four observations (tripletons, doubletons and singletons excluded). Results of beta diversity estimates from this sensitivity analysis were then plotted along with those from the full data set to visualize how similarity measures were affected by exclusion of different compontents of the endophyte community.

The four most abundant and widespread taxa (MOTU numbers 5, 6, 8, 9) were examined for population genetic structure by host and by site using PERMANOVA. First, to obtain a proxy for genotypes within MOTUs, we grouped sequences within each MOTUs into 99% similarity groups using a global clustering analysis (99% MOTU) on the assumption that such groupings would approximate underlying genetic variation. A Bray–Curtis distance matrix for variation within each abundant 95% MOTU was generated. We then examined effects of site, host species and their interaction. We used the 'adonis' function in the R vegan package as an alternative to AMOVA (Excoffier *et al.* 1992), as these two approaches use similar methods of partitioning variance.

Results

We obtained sequence for the ITS rDNA barcoding region for 2079 cultured fungal endophytes collected over 2 years and among three sites in Papua New Guinea lowland rainforest (Fig. 1A). Amplicon length was variable and averaged 628 base pairs. Samples were obtained from 11 different host tree species representing five genera and five plant families (Fig. 1B). The tree species sampled exhibit a range of population densities and spatial distributions in the Wanang FDP (Fig. 2).

DNA sequences were not obtained from all samples either because some cultures did not grow, because of contamination of cultures generated under rudimentary field conditions, or because some cultures were apparently bacterial endophytes (Table 1). Culture rates, the proportion of leaf fragments that produced endophytes, differed among sites (Table 1). In addition, the Wanang 2011 collection had a lower rate of sequencing success than did Ohu and Wamangu collections (Table 1) and resulted in data sets of different size despite approximately equal sampling efforts. The 2010 Wanang data set included 670 ITS sequences representing 54% of the 1241 cultures. The sequences clustered into 60 MOTUs at 95% ITS similarity (Table 1). A total of 1409 sequences were obtained for 2011 collections across the three sites and these clustered into 191 95% MOTUs. A rarefied 2011 data set, standardized to 335 sequences per site, contained 154 endophyte MOTUs in total and an average of 72 MOTUs per site. Species accumulation curves were similar for different sites (Fig. 3). The taxonomic identity, host tree species, location and year collected, and GenBank accession numbers for all 2079 sequenced endophytes are provided in Table S2.

Host-associated community structure

Permutational multivariate analysis of variance (PERMANOVA) was carried out to test for differences in endophyte community composition among host species

Table 1 Culture rate and sequencing rate for endophytic fungal cultures, abundance and richness of MOTUs obtained by site. Culture rate is the percentage of leaf samples from which at least one endophyte culture was obtained. Sequence rate is the percentage of cultured endophytes that resulted in ITS sequences of sufficient quality for further analysis. Abundance is number of sequences produced per site. Richness is the number of different MOTUs obtained. Sequences were rarefied to the smallest sample size (335 sequences) by selecting sequences randomly without replacement within communities

Collection	Culture rate (%)	Sequence rate (%)	Endophyte Abundance	MOTU richness	Rarefied sample richness (n = 335)
Wanang 2010	90	54	670	60	46
Wanang 2011	43	49	394	66	61
Ohu 2011	32	65	335	73	73
Wamangu 2011	61	68	680	127	81

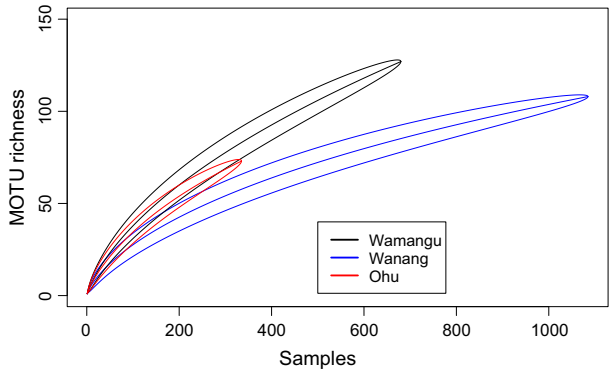

Fig. 3 Fungal endophyte MOTU accumulation curves for each study site in lowland rainforest of Papua New Guinea. Curves represent means of richness values obtained by random draws of a given sample size, without replacement from all MOTUs collected at each site. Standard deviation around the mean is calculated from 100 random permutations of the data. The number of endophyte cultures and sequences differed between sites and years (Table 1).

or genera. For the Wanang 2010 data set, results of PERMANOVA analyses showed that endophyte communities differed significantly among host tree species ($P = 0.001$ and $r^2 = 0.300$ with singletons; $P = 0.001$ and $r^2 = 0.312$ without singletons) and host genera ($P = 0.004$ and $r^2 = 0.161$ with singletons; $P = 0.004$ and $r^2 = 0.167$ without singletons). Similar PERMANOVA models, testing for differences in community composition between host species and between genera, were examined separately for each site sampled in 2011 and for the combined 2011 data set (Table 2). For sites from which the most sequences were recovered (Wanang 2010 and Wamangu 2011) and for the full 2011 data set, statistically significant differences between fungal communities associated with host species and genera were observed (Table 2). In contrast, Ohu 2011 and Wanang 2011 yielded fewer total sequences (Table 1) and did not show significant community differentiation among host groups (Table 2). Even when sampling intensity was sufficient to detect statistically significant differences among hosts, the proportion of variance explained by host identity was relatively low (Table 2). Tests for differentiation of endophyte community composition among sites were statistically significant but also explained relatively little variation ($P =< 0.001$ and $r^2 = 0.075$ with singletons; $P =< 0.001$ and $r^2 = 0.076$ without singletons).

We used permutational tests of multivariate dispersion (PERMDISP) to assess whether heterogeneity of endophyte communities differed among host species and genera. Differences in multivariate dispersion could produce misleading PERMANOVA results. Results of PERMDISP analyses showed that variation in endophytes community composition at Wanang in 2010 was heterogeneous across host species ($P = 0.034$ with and without singletons). Further inspection revealed that only *F. variegata* had a significantly heterogeneous endophyte community compared to four other hosts (*Macaranga punctata*, $P = 0.021$; *Ficus pungens*, $P = 0.025$; *Syzygium longipes*, $P = 0.015$; *Ficus hahliana*, $P = 0.043$). Results of PERMDISP performed for each 2011 site show no statistical evidence of heterogeneity (both with and without singleton MOTUs), and pairwise comparisons confirmed that this pattern was consistent across host taxa within each site. Anderson & Walsh (2013) tested the sensitivity of PERMANOVA to deviations from a balanced sampling design and concluded that results are robust to heterogeneity of group dispersions when sampling is even.

Nonmetric multidimensional scaling (NMDS) provided a visualization of differences in fungal community composition across hosts. The resulting

Table 2 Results of PERMANOVA tests for differences in endophyte community composition between groups of host species and genera. Statistical significance was assessed on 999 permutations of the Bray–Curtis dissimilarity matrix for endophyte community composition in sampled host trees. Model r^2 values represent the proportion of variance explained by groupings of host species or genera. Analyses were carried out on the full data set then replicated with singletons removed to test sensitivity of our results to their inclusion

	Host groups							
	Species		Species (no singletons)		Genera		Genera (no singletons)	
Site	*P*	r^2	*P*	r^2	*P*	r^2	*P*	r^2
Wanang 2010	<0.001	0.300	<0.001	0.312	<0.001	0.161	<0.001	0.167
Wanang 2011	n.s.	0.388	n.s.	0.396	n.s.	0.138	n.s.	0.175
Ohu 2011	n.s	0.350	n.s.	0.375	n.s.	0.148	n.s.	0.169
Wamangu 2011	<0.001	0.437	<0.001	0.470	<0.05	0.183	<0.001	0.203
All 2011	<0.001	0.158	<0.05	0.142	<0.001	0.074	<0.005	0.066

two-dimensional NMDS (stress = 0.221) showed differentiation in ordination space reflecting dissimilarity in endophyte community composition among hosts at the species and generic levels (Fig. 4). Standard deviation ellipses were projected on the ordination plot to visualize multivariate dispersion in community composition by host species (Fig. 4). These ordination results correspond to our PERMANOVA results (Table 2), and the results together suggest slight differences in fungal community composition among host taxa.

We then asked whether differences in community composition between host taxa were correlated with leaf functional traits. Results for permutational ANOVAs testing for correlation between leaf traits and variation in endophyte community composition were similar when traits were evaluated individually or collectively. Except for Ohu 2011, correlations were statistically significant but weak only when SLA, N content and C content were analyzed simultaneously (Table 3).

Analyses aimed at investigating the relative importance of sites, host species identity and leaf functional traits as explanatory variables are presented in Table 4. Variable input order affected model output significantly for host species and traits (Table 4). Functional traits were statistically significant model parameters only when loaded before species and explained a similar proportion of variance as in the previous analysis (Table 3). Host species explained approximately 16% of variance when loaded before traits in the model and 7% when loaded after traits with the difference in r^2 being partitioned among the traits (Table 4). Results did not differ substantially between analyses with or without singleton MOTUs.

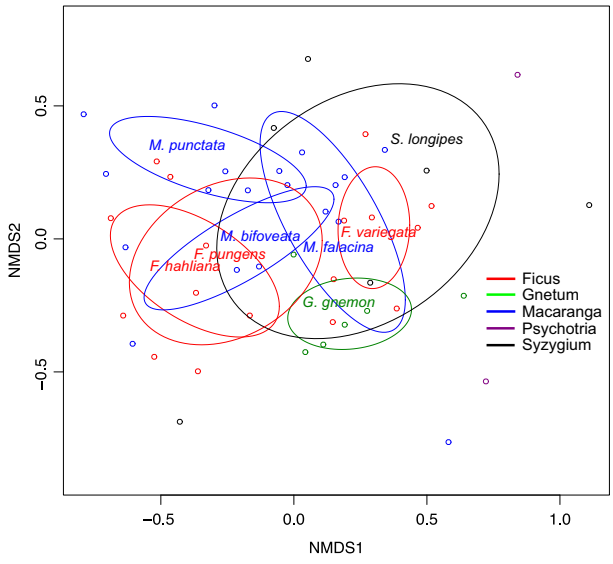

Fig. 4 Local variation of Wanang 2010 endophyte community composition represented by a two-dimensional NMDS (stress = 0.221). Data points represent individual host trees and are coloured according to host genera. Standard deviation ellipses are drawn about host species centroids to indicate location and dispersion in ordination space of endophyte community composition for each host species. An ellipse was not drawn for *Psychotria* for which only two host trees were sampled. *Macaranga* and *Ficus* species are labelled.

Quantitative host–endophyte association webs reveal an endophyte community dominated by a few abundant, widely distributed MOTUs and many rare MOTUs. Examination of endophyte MOTUs across the three sites suggests that many MOTUs were widely distributed (Fig. 5A). Webs grouped by host species for each 2011 site exhibited patterns similar to that of Wanang 2010 (Fig. 5B) in associating a few abundant endophyte MOTUs with all host species and many rare MOTUs isolated from a single host species. This pattern of abundance and rarity extended to the site level (Fig. 5A) where a small set of abundant MOTUs was obtained from all sites with rare taxa often isolated from a single site.

The results of a simple Mantel test showed that dissimilarity of endophyte composition was not significantly correlated with host phylogenetic distance ($P = 0.583$ and $r = -0.016$ with singletons; $P = 0.484$ and $r = 0.006$ without singletons).

Spatial distribution

We explored beta diversity of fungal endophyte communities over distances of 10–100 m between sampled trees in the Wanang FDP and at a regional scale of approximately 100–300 km between sites. At the local scale, Mantel tests for correlation of euclidean distance between hosts and Bray–Curtis dissimilarity of endophyte communities were not significant for individual trees, host species or genera (Table S3). Pairwise comparisons of 2011 collections from Ohu, Wamangu and Wanang yielded Chao–Sorensen values reflecting a high degree of endophyte community similarity among sites (Fig. 6). Excluding endophyte MOTUs from the analyses yielded even higher similarity estimates when rare endophytes were excluded and lower estimates when one or more abundant MOTUs were excluded. Each analysis demonstrated a relatively constant community similarity as a function of distances (Fig. 6). A pattern of low species turnover was consistent in pairwise comparisons of sites, host genera and species (Table 5). Rarefied samples showed also similar but slightly lower values of Chao–Sorensen similarity (Table 5).

Population genetic structure of the four most abundant MOTUs was inferred from ITS sequences for MOTU 5 ($n = 62$), MOTU 6 ($n = 863$), MOTU 8 ($n = 245$) and MOTU 9 ($n = 86$). PERMANOVA for the most abundant MOTU (6) identified significant genetic differ-

Table 3 Distance-based redundancy analysis results evaluating the correlation between host leaf traits and a Bray–Curtis dissimilarity matrix of endophyte communities in sampled trees. Leaf traits are specific leaf area (SLA), foliar nitrogen content and foliar carbon content. Tests were performed for all traits in concert and each trait separately. Significance was evaluated with permutational ANOVA. Model r^2 values represent the proportion of variance in endophyte community composition associated with host leaf traits. Analyses were carried out both with and without singleton endophyte MOTUs.

	All traits		SLA		Foliar Nitrogen		Foliar Carbon	
Site	*P*	r^2	*P*	r^2	*P*	r^2	*P*	r^2
Singletons included								
Wanang 2010	0.003	0.119	0.356	0.023	0.025	0.041	0.083	0.032
Ohu 2011	0.196	0.171	0.630	0.045	0.329	0.055	0.023	0.084
Wamangu 2011	0.002	0.208	0.127	0.058	0.062	0.064	0.006	0.082
Wanang 2011	0.017	0.218	0.071	0.077	0.399	0.052	0.108	0.072
All 2011	0.001	0.084	0.070	0.021	0.019	0.025	0.001	0.042
Singletons removed								
Wanang 2010	0.001	0.122	0.326	0.023	0.030	0.042	0.110	0.031
Ohu 2011	0.172	0.177	0.607	0.044	0.319	0.056	0.021	0.089
Wamangu 2011	0.001	0.229	0.132	0.061	0.052	0.071	0.006	0.090
Wanang 2011	0.025	0.219	0.043	0.084	0.529	0.046	0.107	0.073
All 2011	0.001	0.088	0.094	0.022	0.021	0.026	0.001	0.044

Table 4 Relative variable importance results among site, traits and taxonomy tested with PERMANOVA. Variable order is specified (with SLA, lead carbon and leaf nitrogen shorthand as 'traits') with corresponding *P* and r^2 values below. Statistical significance was assessed on 999 permutations of the Bray–Curtis dissimilarity matrix for endophyte community composition in sampled host trees with the exception of *Psychotria* for which we lacked functional trait information. Values of r^2 represent the proportion of variance explained by individual variables. Analyses were carried out on the full data set then replicated with singletons removed to test sensitivity of our results to their inclusion

	Model							
	Site + traits + species				Site + species + traits			
	With singletons		No singletons		With singletons		No singletons	
Variable	*P*	r^2	*P*	r^2	*P*	r^2	*P*	r^2
Site	0.001	0.087	0.001	0.097	0.001	0.087	0.001	0.097
Species	0.043	0.069	0.046	0.069	0.001	0.156	0.001	0.165
SLA	0.030	0.022	0.044	0.023	n.s.	NA	n.s.	NA
Leaf N	0.001	0.025	0.011	0.028	n.s.	NA	n.s.	NA
Leaf C	0.009	0.041	0.001	0.045	n.s.	NA	n.s.	NA

ences among sites and host species but not for the site*host interaction term (site: $P = 0.001$ and $r^2 = 0.06$; host species: $P = 0.018$ and $r^2 = 0.11$; site*host species: $P = 0.168$ and $r^2 = 0.13$). There was no significant ITS sequence differentiation by site or by host species for the other three most abundant MOTUs.

Discussion

We cultured fungal endophytes to compare the relative influence of host association and dispersal limitation on endophyte community composition in a species-rich lowland tropical rainforest. Endophyte communities differed among host tree species, but variation in community composition was not correlated with host phylogenetic distance. Endophyte community similarity was not related to the spatial proximity of host trees at the scale of 10–100 m, and fungal communities were highly similar among sites separated by hundreds of kilometres across a continuous lowland rainforest in New Guinea.

Host-associated patterns of endophyte distribution

Dissimilarity of endophyte composition was not correlated with host plant phylogenetic distance even

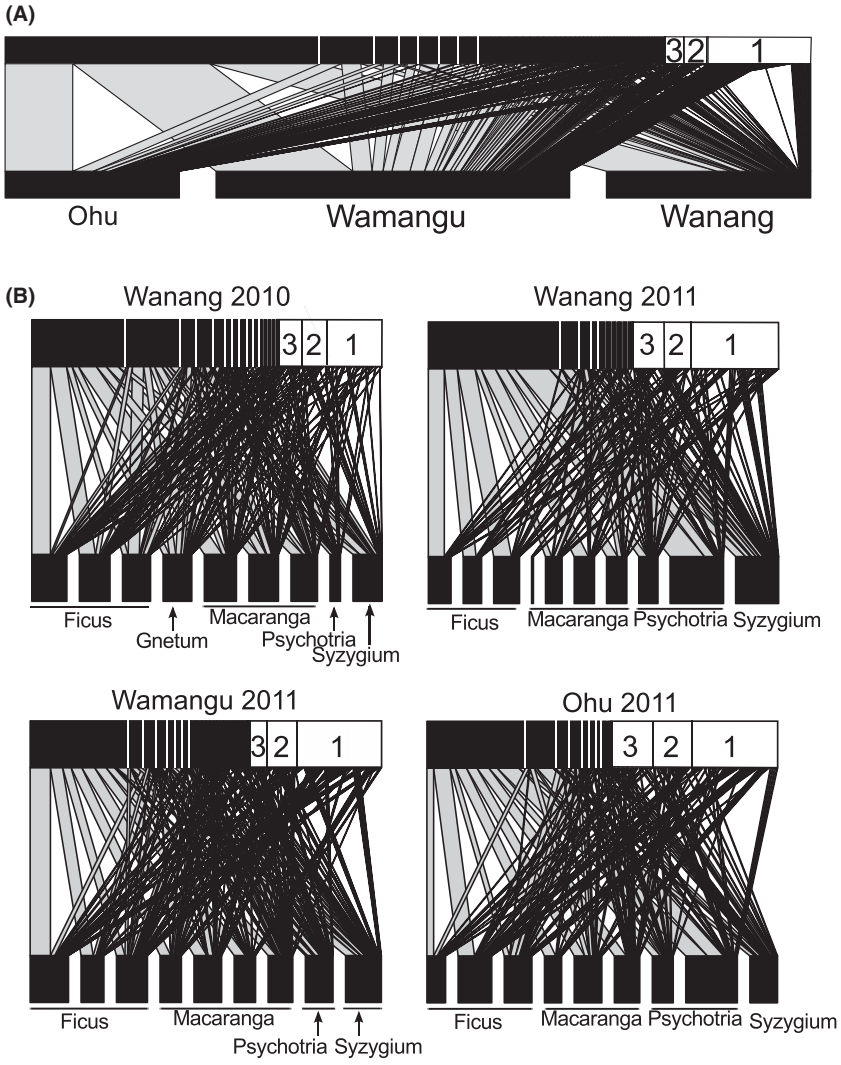

Fig. 5 Bipartite quantitative association webs summarizing the presence of fungal endophyte MOTUs for three sampling sites (A) and for each sampled plant species within individual sites (B). Quantitative host–endophyte association webs (e.g. Lewis *et al.* 2002; Novotny *et al.* 2010) are comprised of an upper row of bars representing abundance of each MOTU and a lower row of bars representing abundance of endophyte communities. The width of lines connecting MOTUs and host species reflect the abundance of each MOTU per site or host tree species. White bars have been superimposed to mark boundaries of bars corresponding to abundant MOTUs. Rare MOTUs are grouped into bins, indicated by numbers representing abundance of singletons (1), doubletons (2) and tripletons (3).

among distantly related seed plants including the broadleaved gymnosperm, *Gnetum gnemon*, and several angiosperms clades. Although previous studies of endophyte community ecology have examined multiple plant lineages (e.g. Arnold & Lutzoni 2007; Higgins *et al.* 2007), ours is the first to explicitly test for a correlation between host plant phylogenetic relatedness and endophyte community similarity. However, endophyte community composition was weakly correlated with phylogenetically conserved foliar traits including specific leaf area, nitrogen content and carbon content (Whitfeld *et al.* 2012b). Leaf traits accounting for up to 22% of the variation among endophyte communities (Table 3) suggest that leaf quality influences habitability. It is possible that additional features of the host neighbourhood, local environment (Arnold & Herre 2003; Pan *et al.* 2008) and life history traits other than those we measured are more strongly associated with endophyte community composition than the foliar traits we evaluated. PERMANOVA results comparing the explanatory power of host location, species identity and leaf traits are consistent with this notion given that traits explained no more variance than did species identity when sequentially loaded in

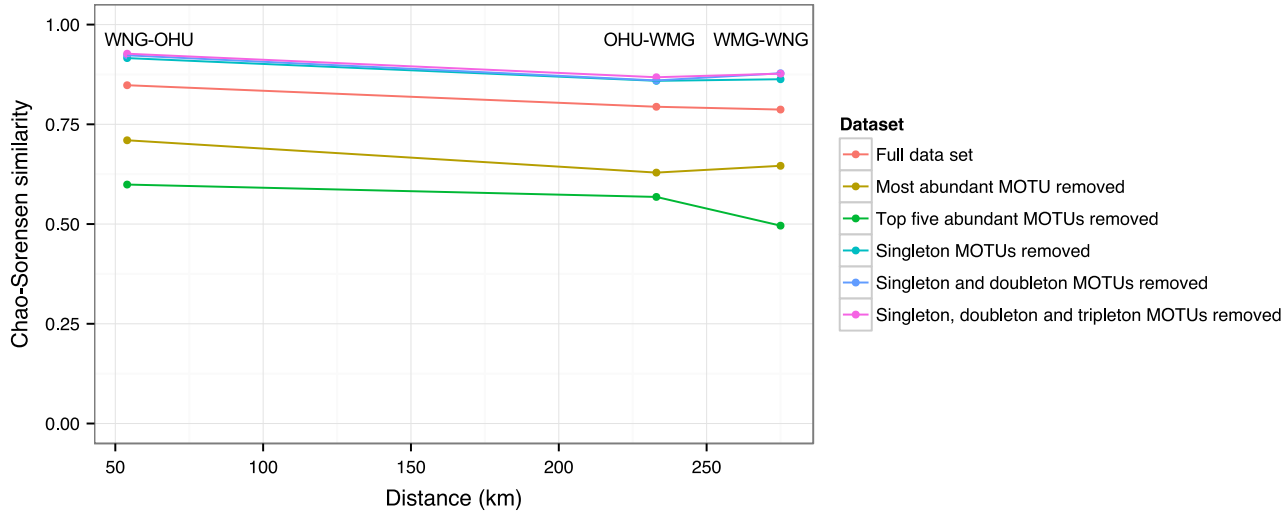

Fig. 6 Pairwise endophyte community Chao–Sorensen similarity values plotted against the distance between sites (km) for Ohu (OHU), Wamangu (WMG) and Wanang (WNG) 2011 collections. Values represent community similarity between pairs of sites. Values for different data sets are connected by lines for visualization only and no correlations of community similarity and spatial distance are implied.

Table 5 Similarity of endophyte community composition for pairwise comparisons of sites sampled in 2011; Ohu (OHU), Wanang (WNG), Wamangu (WGU). Chao–Sorensen values, for endophyte community similarity between sites and across all host species, across species within the genera *Ficus* and *Macaranga*, and for each host species sampled at all sites in 2011, are given. Values for the full data set (not rarefied) and rarefied data are presented for comparison. Values in the '*n*' column denote sample size of number of sequences for rarefied data sets. For example, the community matrix of endophytes cultured from *Ficus* for each site was randomly subsampled to produce data sets of 107 endophyte sequences

		WNG-OHU		WGU-OHU		WNG-WGU	
Scale	*n*	Rarefied	Not rarefied	Rarefied	Not rarefied	Rarefied	Not rarefied
Sites	335	0.836	0.849	0.804	0.787	0.790	0.794
Ficus	107	0.923	0.716	0.707	0.716	0.827	0.786
Macaranga	94	0.756	0.753	0.839	0.736	0.780	0.716
Ficus hahliana	25	0.719	0.720	0.461	0.471	0.616	0.721
Ficus pungens	30	0.605	0.681	0.626	0.648	0.645	0.548
Ficus variegata	37	0.669	0.546	0.600	0.583	0.502	0.655
Macaranga bifoveata	27	0.738	0.689	0.443	0.612	0.305	0.637
Macaranga punctata	36	0.557	0.54	0.544	0.654	0.496	0.54
Psychotria micrococca	69	0.863	0.923	0.564	0.557	0.611	0.611
Syzygium longipes	37	0.869	0.855	0.848	0.695	0.601	0.761

the analysis. Host tree identity explained a substantial proportion of variation among endophyte communities (up to 44%) and could possibly limit the set of fungal taxa capable of colonizing particular hosts.

A few generalists were abundant in every host tree species and each site we examined. This same pattern was observed across two sampling years at Wanang despite somewhat different sampling methods. Observed differences among endophyte communities associated with different host species resulted primarily from differences in the presence of rare MOTUs (e.g. singletons and doubletons). Thus, it appears that variation in endophyte composition among hosts is influenced by quantitative differences in the relative abundance of generalists and the presence or absence of rare MOTUs in particular hosts. Results of PERMANOVA provided statistical evidence to support the hypothesis that tree species and genera support somewhat different endophyte communities despite overlap in widespread and abundant taxa.

Our observation of endophyte occurrence patterns in rainforest trees agrees with findings in tropical trees (Cannon & Simmons 2002; Arnold & Lutzoni 2007), grasses (Higgins *et al.* 2011, 2014) and seeds (Kluger *et al.* 2008), suggesting that endophytic fungi are generalists with respect to host associations, at least at the level of MOTUs. We did observe host-associated population genetic differentiation in our most abundant MOTU, a finding that contrasts with that of Oono *et al.* (2014). Greater host specificity has been demonstrated in tropical saprophytic fungi (e.g. Gilbert & Sousa 2002; Augspurger & Wilkinson 2007; Kembel & Mueller 2014), and for epiphytic fungi (Kembel & Mueller 2014).

Spatial patterns of endophyte distribution

We found no correlation between endophyte community composition and Euclidean distance between host trees, a result suggesting a pattern of widespread occurrence at the local scale of 10–100 m. The remarkable absence of spatial patterns in the composition of endophyte communities at a regional scale of 100–300 km among individual trees, host species and host genera suggests either that our sample size was insufficient to detect differences or that dispersal limitation does not play a significant role in shaping the distribution of endophyte species across large distances. However, genetic differentiation among regional populations of the most abundant MOTU6 suggests the intriguing possibility that, in contiguous forest without strong environmental gradients, fungal endophyte species populations might reach a rarely observed mutation–migration equilibrium.

Although our sampling effort may not have been sufficient to capture differences in spatial distribution and relative abundance of host tree species, we observed a remarkably even distribution of endophyte community composition across tree species exhibiting a broad range of population density and spatial pattern. For example, *Gnetum gnemon* is abundant and shows an even distribution across the Wanang plot, *Ficus hahliana* is abundant but is restricted to drainages, and *Ficus pungens* is sparse and found primarily in tightly aggregated patches (Fig. 2). Host density and degree of spatial clustering could influence endophyte distribution and yet we could identify no patterns consistent with this hypothesis. Taken together, the results of our fine-scale spatial analysis support the conclusion that differences in endophyte community composition between tree species may be shaped more by host associations than by an underlying process of dispersal limitation.

Patterns of endophyte community turnover in relation to environmental gradients have been consistent in the literature until now. Previous studies at larger spatial scales found substantial differences in endophyte community composition in relation to abiotic environment and differences in host community composition (U'Ren *et al.* 2012; Zimmerman & Vitousek 2012; Peay *et al.* 2013; Higgins *et al.* 2014), suggesting that turnover in many fungal endophyte communities is strongly affected by ecological gradients and turnover of plant species. Additionally, recent studies of soil fungi have found that variation in both fungal richness and composition is tied to changes in host plant composition and climatic variables between sites (Gao *et al.* 2013; Tedersoo *et al.* 2014; Prober *et al.* 2015). The absence of significant abiotic differences (e.g. climate, soil, elevation) among our regional sites and the broad distribution of host species uniquely allowed us to ask whether dispersal limitation alone could influence endophyte community similarity. Our findings suggest low beta diversity over the distances of hundreds of km in a contiguous New Guinea rainforest. This result is a striking contrast to evidence of decreasing endophyte community similarity with increasing distance between trees (Arnold 2003). We hypothesize that widespread host species occupying a large, contiguous area with a relatively homogenous abiotic environment could allow for dispersal and homogenization of endophyte communities over time.

Notably, low beta diversity is evident among other taxa in this region including herbivorous insects of the same rain forest trees that we studied (*Ficus*, *Macaranga* and *Psychotria*) (Hulcr *et al.* 2007; Novotny *et al.* 2007). Interestingly, widespread herbivore species with a relatively even distribution across the landscape were dietary specialists for the most part (Novotny *et al.* 2002). The low beta diversity of fungal endophytes we observed in the same lowland rainforests could possibly be explained by long distance spore dispersal. If generalism is predominant, as others (Higgins *et al.* 2011, 2014) have observed, and host availability is not limiting, movement through a contiguous lowland rainforest lacking any significant dispersal barriers is expected over time. Alternatively, these sites could represent discrete communities that are in equilibrium in regard to species composition, an inference supported by evidence of underlying genetic structure associated with both hosts and sites in the most abundant MOTUs of our study. Although this pattern was observed only in the most abundant MOTUs of the four we investigated and is based on ITS sequence only, the result is consistent with that of Oono *et al.* (2014) for temperate endophyte populations in which genetic structure due to spatial distance among populations was detected. Further sampling and loci will shed light on whether widespread generalist endophyte taxa are in migration–drift equilibrium.

One possible limitation of our study relative to metagenomic approaches (e.g. Zimmerman & Vitousek 2012) is that our data set is small and may not fully capture local species richness, as accumulation curves show no sign of an asymptote, which is also true of other studies of diverse fungal endophyte communities (Arnold & Lutzoni 2007). Culture-independent and culture-based methods (Allen *et al.* 2003; Arnold 2007; Higgins *et al.* 2011) will be valuable in future studies of PNG forest endophyte communities as each method has potential biases and strengths in estimating fungal diversity (Tedersoo *et al.* 2010).

Conclusions

The results of our analyses and data visualizations suggest that host associations play a role in shaping fungal endophyte community composition in rainforest trees. Weak but significant patterns of host-associated community similarity were found despite limited sampled of host species differing in spatial distributions, population density, phylogenetic relatedness and leaf functional traits. Patterns of endophyte occurrence, both within sites and in aggregate, were characterized by a small set of abundant host generalists and many rare taxa. Beta diversity of fungal endophytes was low between sites spanning hundreds of kilometres and situated in a continuous and abiotically homogenous lowland rainforest. This finding suggests that in the absence of environmental gradients and changes in host plant community composition, fungal endophyte communities could be highly similar across spatial scales and that this similarity is due to a few common and widely dispersed fungal taxa. The pattern of low regional beta diversity, if consistent among rainforests, could translate into lower global estimates for numbers of fungal species (Arnold *et al.* 2000; Blackwell 2011). Considered together, our conclusions support the notion that host associations play a weak but relatively more important role than dispersal limitation in shaping the distribution of fungal endophyte communities in terms of both local variation and regional turnover.

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J.V. performed the statistical analyses and drafted the manuscript. G.W. coordinated the fieldwork. G.M. oversaw the fungal culture collection and DNA sequencing. G.M. and G.W. supervised data collection and analysis, and wrote part of the manuscript. J.V., G.W. and G.M. interpreted results and edited the manuscript.

Data accessibility

DNA sequences and associated metadata: Genbank Accessions KR015110–KR016829, Table S2.

All data files used for analyses and R analysis scripts: Dryad doi:10.5061/dryad.7q1b2.

Supporting information

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

Table S1. Host tree species sampled per site and year for data collections at Ohu, Wamangu, and Wanang in 2010 and 2011.

Table S2. Information on MOTU (95% similarity), host plant species, UNITE blast results, and associated Genbank accession number for every cultured endophyte collected in 2010 and 2011 in Papua New Guinea.

Table S3. Results of Mantel tests for correlation between endophyte community composition and distance between sampled trees at Wanang in 2010.