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ORIGINAL PAPER

Endophytic fungi from Peruvian highland and lowland habitats form distinctive and host plant-specific assemblages

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Abstract Biodiversity and biogeography of leaf-inhabiting endophytic fungi have not been resolved yet. This is because host specificity, life cycles and species concepts, in this heterogeneous ecological guild of plant-associated microfungi, are far from being understood. Even though it is known that culture-based collection techniques are often biased, this has been the method of choice for studying fungal endophytes. Isolation of fungal endophytes only through culture-based methods could potentially mask slow growing species as well as species with low prevalence, preventing the capture of the communities' real diversity and composition. This bias can be partially resolved by the use of cultivation-independent approaches such as direct sequencing of plant tissue by next generation techniques. Irrespective of the chosen sampling method, an efficient analysis of community ecology is urgently needed in order to evaluate the driving forces acting on fungal endophytic communities. In the present study, endophytic ascomyceteous fungi from three different plant genera (*Vasconcellea microcarpa*, *Tillandsia* spp., and *Hevea brasiliensis*)

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distributed in Peru, were isolated through culture-based sampling techniques and sequenced for their ITS rDNA region. These data sets were used to assess host preferences and biogeographic patterns of endophytic assemblages. This study showed that the effect of the host's genetic background (identity) has a significant effect on the composition of the fungal endophytic community. In other words, the composition of the fungal endophytic community was significantly related to their host's taxonomic identity. However, this was not true for all endophytic groups, since we found some endophytic groups (e.g. Xylariales and Pleosporales) occurring in more than one host genus. Findings from this study promote the formulation of hypotheses related to the effect of altitudinal changes on the endophytic communities along the Eastern Andean slopes. These hypotheses and perspectives for fungal biodiversity research and conservation in Peru are addressed and discussed.

Keywords Plant-associated microfungi · Fungal biodiversity research · Fungal ecology · Andes-Amazon region of Peru · Elevation gradient · Sustainable biodiversity research

Introduction

Ecology and diversity of fungal endophytes

Leaf-inhabiting endophytic stages of fungi are surrounded by living plant tissue that provides a more stable micro-environment than the external abiotic surroundings (Körner 1989). Usually host plant foliage is very little affected (i.e., destroyed) by such fungal infections, however various beneficial and detrimental effects for the host may occur due to indirect multitrophic interactions (Devarajan and Suryanarayanan 2006; Faeth and Saari 2012). Living leaves are inhabited by species-rich endophytic communities (Lodge et al. 1996), representing one of the most complex fungal communities in terrestrial ecosystems (Saikkonen et al. 2010; White and Bacon 2012). This complexity is particularly high for the non-systemic foliar tree endophytes, that consist of mutualists, facultative insect pathogens, parasites and saprobes, latent and weak plant pathogens or simply of dormant erratic colonizers of an inappropriate host plant (Rodriguez et al. 2009; Saikkonen et al. 2010; Bills et al. 2012; Hofstetter et al. 2012). Fungal endophytes have been found to interact with microorganisms on the leaf surface (e.g. Vega et al. 2009) and on dead fallen leaf litter (Osono 2011; Unterseher et al. 2013).

Habitat—host plant—and tissue specificity are intensively explored in fungal biodiversity research. This subject is experiencing a change of trend by the latest developments in molecular technologies (Kubartova et al. 2012; Danielsen et al. 2012; Cordier et al. 2012a). Next generation sequencing of environmental samples as well as improvement in cultivation methods (Collado et al. 2007; Unterseher and Schnittler 2009; Tormo et al. 2012; Unterseher et al. 2013) increase both, the quantity and quality of the available data. However, verification of strictly DNA-based species identification against sequenced type strains is more important than ever (Ko et al. 2011).

Comparative next generation sequencing (NGS) studies have shown that fungal phyllosphere assemblages of *Quercus macrocarpa* differed between urban and rural sites within the same locality in addition to significant seasonal composition changes (Jumponen and Jones 2009, 2010). Similarly, NGS studies of phyllosphere fungi from *Fagus sylvatica* indicated an effect of the genetic variation of host trees on the composition of their phyllosphere fungal species (Cordier et al. 2012a). The same study also detected a

significant influence of climatic variables on fungal species abundance and composition along an elevation gradient (Cordier et al. 2012b).

Arnold et al. (2007) surveyed fungal endophytes from hosts distributed from the North American tundra to the Neotropics, and observed significant effects of latitude on species richness and composition. In another study, it was shown that both geography and host-plant identity shaped the community composition of endophytes in cuppresaceous host plants (Hoffman and Arnold 2008). Recently a significant overlap in OTU composition of cultivable leaf endophytes from European *Populus tremula* (Albrectsen et al. 2010) and Central Asian *P. euphratica* was reported (Unterseher et al. 2012). These studies suggest that host plant identity is a major driving factor for the dynamics of endophytic communities. Additional studies have shown that endophytic fungal communities are influenced at both small-scale and short-term (Fröhlich and Hyde 1999; Unterseher et al. 2007) and large-scale and long-term regimes (U'Ren et al. 2012).

Due to the high coverage of sequences per taxon and sample, culture-independent NGS studies of the phyllosphere hold great promises in detecting unbiased fungal assemblages. However, these techniques do not yet allow to distinguish between fungal endophytes and fungal epiphytes (Jumpponen and Jones 2009; Cordier et al. 2012a, b). This stands in clear contrast to the pervasive paradigm in "endophytology", that is the isolation of living strains from surface-sterilized leaf fragments (Schulz et al. 1993). Most recently, Unterseher et al. (2013) compared fungal NGS data (Cordier et al. 2012a) against culture-based species lists from the same tree species (Unterseher and Schnittler 2010) and concluded that culture-based studies could indeed reveal "real" biodiversity patterns of fungal forest endophytes. The use of improved cultivation techniques (Paulus et al. 2003; Collado et al. 2007; Unterseher and Schnittler 2009), multi-gene phylogenetic analysis (Gazis et al. 2011) and various in vitro experiments are still important methods to generate solid reference information that allows the assessment of microfungal biology, ecology and morphology (Gams et al. 2012). The availability of living strains allows the testing of various hypotheses that could emerge from cultivation-independent 'omics' studies (Giovannoni and Stingl 2007).

Fungal biodiversity research in Peru

The Republic of Peru is one of the largest countries in South America. The country's geography is characterized by coastal pacific regions, Andean highlands including the Altiplano plateau and tropical lowland Amazonian rain forests, the latter covering ~60 % of the country's area. The unique combination of such diverging landscapes has created many different ecosystems in this biodiversity-mega-rich country that contains high levels of endemism (Swenson et al. 2012). For instance, in the Manu National Park in Peru's Madre de Dios region alone, 693 tree species have been identified (Pitman et al. 2002).

Apart from the pioneering research on fungal endophytes of rubber trees (*Hevea* spp.) which revealed an enormous hidden diversity (e.g. Gazis and Chaverri 2010; Gazis et al. 2011), few studies involving fungal biodiversity of Peru have been published so far. Nevertheless, species (Samuels and Ismaiel 2009; Poldmaa 2007; Chaverri et al. 2011), families (Raja et al. 2012) and even an entire new fungal class (the Xylonomycetes; Gazis et al. 2012) have been described from Peruvian samples. This highlights the enormous potential for future mycological studies in the country (Palin et al. 2011; Bascom-Slack et al. 2009; Smith et al. 2008; Berkov et al. 2007) that additionally has considerable ethnomycological tradition (Trutmann 2012) and various activities in collecting and growing edible mushrooms (Chimey Henna and Holgado Rojas 2010).

It becomes more and more important for current and future biodiversity projects to implement an umbrella-like structure unifying pure research and scientific novelties with societal, economic and political aspects (e.g. Barnard et al. 1998). So far, many current initiatives against poverty, devastating environmental changes and corruption in developing countries are based mainly upon politics, trade and industry. Thereby, the value of biodiversity and unmodified natural resources for local, regional or global benefits and markets let alone for peace and freedom have been widely disregarded (http://www.parkswatch.org/parkprofiles/pdf/ampf_eng.pdf [accessed Dec 2012]), but see Kumar (2012).

In this spirit, the first delegates of a multidisciplinary research team of experts in animal taxonomy, molecular biodiversity, ecosystem services and species preservation visited Peru in December 2011 aiming to discuss the launching of a long-term collaborative biodiversity research initiative. During this mission, sampling trips were conducted in collaboration with authorities of the Province of Chachapoyas and several Andean Villages, the University of Chachapoyas, the "Instituto Nacional de Innovación Agraria (INIA)–Chachapoyas", non-governmental (non-profit) organizations and environmental, Christian and indigenous organizations.

The present study used (I) endophyte data from the two plant genera *Vasconcellea* and *Tillandsia* which were collected during the above mentioned journey and (II) endophyte data from a third genus (*Hevea*) and from different Peruvian sampling sites (Gazis and Chaverri 2010). In addition to ITS sequencing and phylogenetic analysis, methods of community ecology were used such as species richness analysis and multivariate statistics. The hypotheses of (I) host-plant specificity in fungal endophytes and (II) the negative relationship between fungal diversity and altitude were assessed.

Materials and methods

Host plants and study sites

Leaves of the native plant species *Vasconcellea microcarpa* were removed from 10 plants at various locations around the village of Granada located ~3,000 m. a.s.l. in the Chachapoyas Region of Northern Peru (Fig. 1; Table 1). The large, healthy looking leaves of *V. microcarpa* were cut into smaller pieces for better transportation. *V. microcarpa* is also called highland papaya ("Maushan") and belongs to the Caricaceae (Brassicales), a small family of vascular plants comprising 6 genera and 35 species. *Vasconcellea* is the largest genus with an estimate of 21 species. The genus probably originated in the South American highlands (van Droogenbroeck et al. 2004; Scheldeman et al. 2007). Some species of the genus including *V. microcarpa* can be considered as genetically rich untapped crops (van Droogenbroeck et al. 2004), whose tasty, high quality fruits and proteolytic enzymes have not yet been object of economic focus in contrast to the much more important relative *Carica papaya* (Duke 1983; FAOSTAT 2012). Following recommendations from colleagues of INIA, who aim at investigating the potential of *V. microcarpa* for sustainable crop plantations in lesser-developed Andean regions (García Guarniz, unpublished project draft) we decided to investigate the endophytic diversity harbored within the leaves of this host plant.

During a second field trip within the same week, ten individuals each of three *Tillandsia* species (*Tillandsia usneoides*, *T.* cf. *purpurea* and *T.* cf. *cacticola*) were removed from their supporting trees (*Acacia* sp.) distributed along a winding road in open south-side landscape 1 km outside of the City of Magdalena (Fig. 1). The genus *Tillandsia* belongs to the Neotropical monocotyledonous Bromeliaceae; most of its species live as rootless

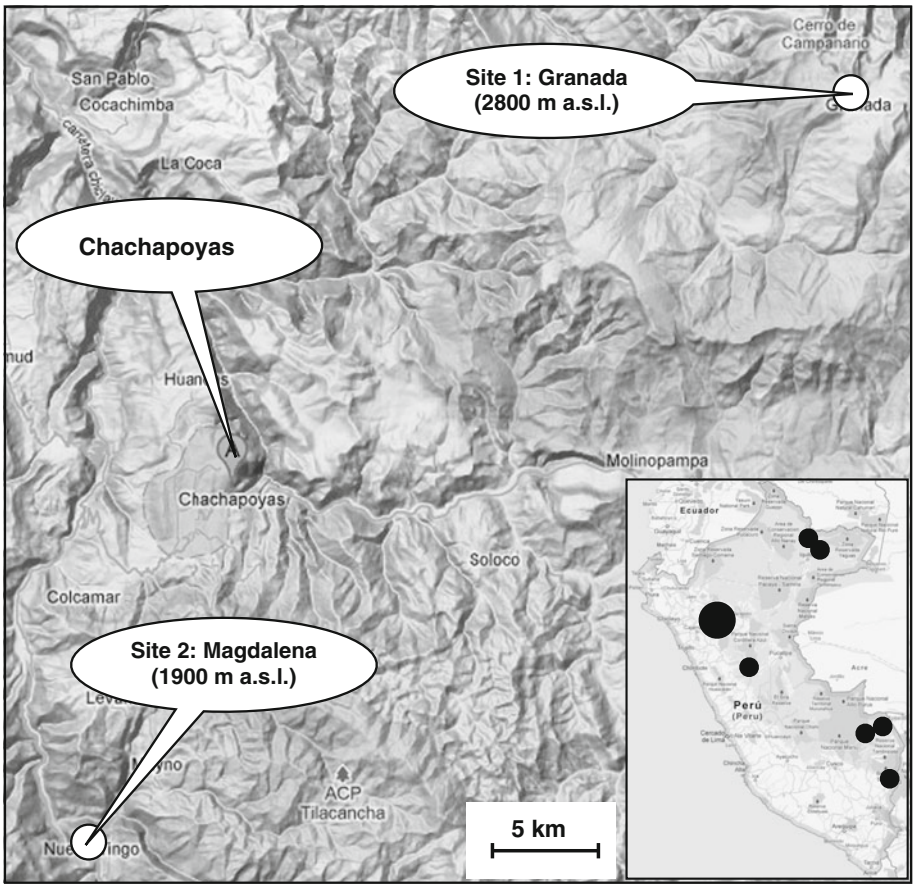


Fig. 1 Sampling sites in Peru. At site 1, *Vasconcellea microcarpa* was sampled. At site 2 the three *Tillandsia* species *T. usneoides, T.* cf. *purpurea* and *T.* cf. *cacticola* were sampled. The *large black dot* in inlay shows the position of the three sites. The *smaller dots* in inlay show additional sampling sites of another research project (e.g. Gazis and Chaverri 2010; Gazis et al. 2011; Chaverri et al. 2011), from which data were used in the present paper. Further details of these sampling sites are available in the respective publications and in the online supplementary of this study. Both maps were modified from Google Maps

atmospheric epiphytic plants in very different habitats, from coastal deserts to tropical montane cloud forests (e.g. Barfuss et al. 2005). *Tillandsia* is a relative large genus with around 550 species and a strongly unstable taxonomy (Zizka et al. 2009).

One day after sampling, preparation of leaves for isolation of endophytes was conducted under semi-sterile conditions. Leaf pieces were carefully but thoroughly washed with bottled drinking water, drained and shook in 70 % ethanol for 5 min and dried in still air in-between four semicircular placed blazing alcohol burners. Leaf pieces, 4 mm in diam., were cut with flamed boring tool and placed into the wells of 48-multiwell plates containing 1.5 % malt extract agar (MEA + 0.1 g/l Tetracycline + 10 µl/l Cyclosporin A). The multiwell plates were sealed with Parafilm and transferred to the University of Greifswald for further laboratory analyses.

Table 1 Description of the sampling sites. Abbreviations contain information about the host plant

Sample.id	Location	Host.gen	Host.spec	Country	Elevation	Lat.dec	Long.dec	X.easting	Y.northing
S.01	GN	VAS	VASmic	Peru	3000	−6.05	−77.58	214.4	9330.59
S.02	MG	TIL	TILusn	Peru	1840	−6.38	−77.9	179.15	9293.89
S.03	MG	TIL	TILpur	Peru	1840	−6.38	−77.9	179.15	9293.89
S.04	MG	TIL	TILcac	Peru	1840	−6.38	−77.9	179.15	9293.89
S.05	FL	TIL	TILusn	USA	50	7.19	−81.34	462.77	794.61
S.06	CM	HEV	HEVbra	Cameroon	170	4.16	9.23	525.53	459.28
S.07	IB	HEV	HEVbra	Peru	270	−11.41	−69.49	446.55	8738.64
S.08	IQ	HEV	HEVbra	Peru	105	−3.25	−72.91	732.24	9640.53
S.09	LA	HEV	HEVbra	Peru	280	−12.51	−70.06	384.82	8616.82
S.10	MS	HEV	HEVbra	Peru	90	−3.62	−72.25	805.52	9599.41
S.11	MX	HEV	HEVbra	Mexico	15	17.98	−93.39	458.71	1988.02
S.12	PP	HEV	HEVbra	Peru	400	−14.22	−69.17	481.66	8427.94
S.13	TC	HEV	HEVbra	Peru	525	−8.19	−76.54	330.35	9094.37

Their geographic position is shown as decimal GPS (Lat.dec and Long.dec) and Cartesian coordinates (X.easting and Y.northing)

TILusn Tillandsia usneoides, TILpur T. cf. *purpurea*, *TILcac T.* cf. *cacticola*, *VASmic Vasconcellea microcarpa, HEVbra Hevea brasiliensis* and site information *GN* Granada, *MG* Magdalena, *FL* Florida, *CM* Cameroon, *IB* Iberia, *IQ* Iquitos, *LA* Los Amigos, *MS* Madre Selva, *MX* Mexico, *PP* Tambopata, *TC* Tocache

Cultivation and morphological identification

Isolation of fungal strains in Germany started a week after the first preparation. Small pieces of mycelia were removed from the cultivars with a fine forceps under sterile conditions and transferred into new axenic MEA-containing Petri Dishes. Emerging fungal colonies were grouped to morphotypes (Lacap et al. 2003, Unterseher and Schnittler 2009). Sporulating cultures were identified using various identification resources (e.g. Seifert et al. 2011; Domsch et al. 2007; Ellis 1971, 1976; Ellis and Ellis 1988; Sutton 1980).

DNA extraction, PCR and ITS sequencing

Fungal tissue with as little agar as possible was removed from 3 to 6 weeks old axenic cultures with an inoculation loop, transferred into a 2 ml safe-seal tube and homogenized in a Ball Mill Retsch for 3 min at 30 Hz with two steel beads (∅ 3 mm). DNA was extracted using the MasterPure Yeast DNA Purification Kit (Epicentre Biotechnologies, Madison), following the manufacturer's instructions. PCR of the fungal *ITS1-5.8S-ITS2* region with the primer pair V9G/ITS4 followed standard protocols (Unterseher and Schnittler 2009). The probes were shipped as unpurified PCR products to GATC Biotech (Konstanz, Germany) for sequencing. Newly generated sequences are accessible from GenBank (HE820744–HE820902).

External data

In order to increase the analytical resolution of the study we included sequences from further locations and plant species in and outside of Peru (Fig. 1). One data set was from a published study (U'Ren et al. 2012) which contained 26 ITS sequences from *Tillandsia usneoides* endophytes from Florida, USA (JQ759930–JQ760131). The second much larger data set was provided by the coauthors RG and PC and originally contained 950 ITS sequences of cultivated ascomycetous leaf endophytes of *Hevea brasiliensis* and *H. guianensis* from six locations in Peru (two of them from plantations) and from one sampling site each in Mexico and Cameroon (plantations). Sequences from the project "Systematics of fungi associated to wild rubber (*Hevea* spp.) trees in the amazon basin" (led by P. Chaverri) are available under accession numbers JQ905630–JQ905831, HQ022302–HQ022514, and FJ884070–FJ884196.

Sampling depth of *Hevea* endophytes per site surpassed that of own and Florida endophytes with an average of 120 sequences (range 86–338). We decided to use comparable sampling intensities for all sites under investigation. Therefore, subsamples of 30 sequences per site were randomly drawn from the *Hevea* data sets to conduct all subsequent analyses. The entire procedure (subsampling and statistical analysis) was repeated three times in order to check for the possibility of randomly distorted assemblages and differing community signals. The observed biodiversity patterns and community signals remained similar for all replicates. Results and supplementary files are therefore based on the first replicate only. Details of subsampling, sequence management, phylogeny and community statistics are explained in the supplementary R-source file (Supplementary material 1). This file also describes, how to call external applications, such as Fungal ITS extractor (Nilsson et al. 2010), MAFFT (Katoh and Toh 2008) or MrBayes (Huelsenbeck and Ronquist 2001) from within the R environment (R Development Core Team 2012).

OTU delimitation, sequence-based taxonomy and phylogenetic analysis

The Fungal ITS extractor was used to remove the *SSU*/*LSU* flanking regions and the intermediate *5.8S* from the ITS sequences. For OTU delimitation pairwise similarities among ITS1 sequences were calculated using Local BLAST (ftp://ftp.ncbi.nih.gov/blast/executables/release/2.2.26 [accessed Dec. 2012]) with the parameters '-m8 -r2 -G5 -E2′. The *R* function 'simMatrix' (Persoh et al. 2010) was applied to transform the calculated pairwise similarities into a similarity matrix, and a hierarchical cluster analysis (*R* function 'hclust') was conducted to group similar ITS1 genotypes to OTUs by the method 'average linkage' using a threshold of 97 % ITS1 similarity (excluding 5.8S and ITS2). At first glance this seems contradictory to own findings (99 % ITS similarity in Gazis et al. 2011 and 100 % in Unterseher et al. 2012). However, in both previous studies, the entire ITS1-5.8S-ITS2 region was used in contrast to the present study, where usage of only the most variable ITS1 allowed for reduction of the threshold to 97 %. Exemplary tests with data from Gazis et al. (2011) proved to be mostly consistent with their findings (data not shown). We therefore relied on our OTU clustering for further analyses although knowing that improvements are needed towards a "next generation OTU clustering" in fungal biodiversity studies (Powell et al. 2011; Powell 2012; Vrålstad 2011, Nilsson pers. comm.).

BLAST (Altschul et al. 1990) and the CBS fungal identification system (http://www.cbs.knaw.nl/Collections/BioloMICSSequences.aspx?file=all [last accessed Dec. 2012)) were used to validate identification of sporulating Peruvian *Vasconcellea* and *Tillandsia* endophytes and to assign taxonomic information to as many of the sterile isolates as possible. Though species names given in the text and supplementary files were drawn from very well curated databases or reliable working groups (CBS, AFTOL) the sequence-based identification of OTUs should be interpreted with caution since they are not checked thoroughly against type species sequences.

Sequences were visualized and manually edited when needed with Mesquite (Maddison and Maddison 2011) and aligned with MAFFT version 6 using the E-INS-i strategy (Katoh and Toh 2008). ITS phylogeny was constructed under Bayesian inference (BI) using MrBayes V3.2.1 and included an appropriate model for minimum evolution selected from the 24 models implemented in MrModeltest 2.1 (Nylander 2004). Each Bayesian analyses used one cold and three heated Monte Carlo Markov chains in two simultaneous runs (default settings) with a temperature of 0.05. Number of generations, sample frequencies and burn-in ratio were set at 10 Mio, 10 and 0.25, respectively. Clade support was assessed with posterior probabilities.

Analysis of community composition

A "sample-species matrix" was compiled with presences or absences of an OTU from a given sample (site). Additionally, a sample-environment matrix was compiled with information about host plant identity, location and altitude (Supplementary material 2). With these two main elements, community composition (i.e., substrate preferences) was analyzed with R using both non-metric multidimensional scaling (NMS or NMDS) based on Bray–Curtis dissimilarities and detrended correspondence analysis (DCA). The explanatory power of environmental variables (host genus, geographic location, and elevation) was tested with a permutational multivariate analysis of variance using Bray–Curtis distance (Oksanen 2011), a multivariate version of analysis of variance (Wang et al. 2012) and a Mantel test implemented in the R-package "BiodiversityR". More details of statistics and interpretation of the data are given in the supplementary R-source file (Supplementary material 1).

Species richness analysis

Analysis of OTU richness used mathematically smoothed species accumulation curves to display the accumulation of "species" with increasing number of records (Gotelli and Colwell 2001). By analyzing the shape of the curves (e.g. initial slope, approaching an asymptote or not), it was possible to evaluate basic patterns of species richness (e.g. sampling intensity). Tests for correlation of species richness with environmental variables were performed by comparing calculated species richness of different sites at the largest common sample size. Species richness estimators Chao, Jackknife 1, and Bootstrap (e.g. reviewed in Colwell and Coddington 1994) were calculated with *R*. Thereby, an extrapolated species richness is considered a serious estimation only if the value of the respective estimator remains stable (i.e., if the respective estimator's curve shows a stable asymptote for a considerable part at the right end of the plotted curve).

Results

The ITS region of 130 endophytic strains from *V. microcarpa* and *Tillandsia* spp. was sequenced and grouped into 55 ascomycete operational taxonomic units (OTUs). Together with all external sequences 155 OTUs from 390 isolates were distinguished (Supplementary material 2, "esm02_peru_ascos_main.xls" spreadsheet "OTU_Matrix_rep1").

Species richness analysis revealed divergent species accumulation curves from *Hevea* spp (Fig. 2, dashed curves), *Tillandsia* spp. (dotted curves) and *Vasconcellea microcarpa* (solid curve). Based on the largest common sampling size (dashed vertical line in Fig. 2) species richness was lowest for the endophytic assemblages of *V. microcarpa*. Species

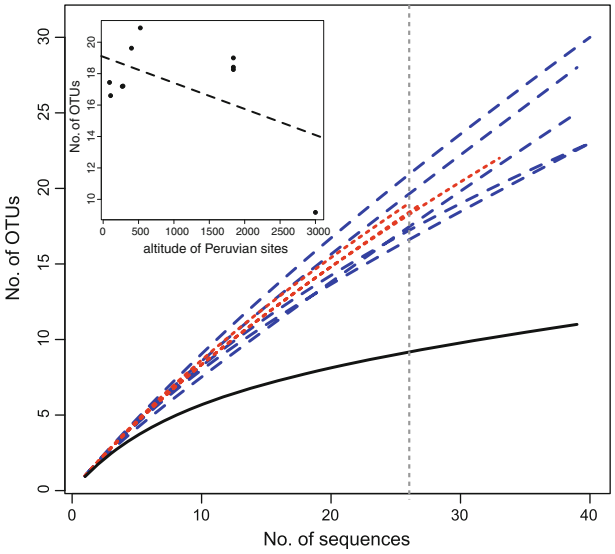


Fig. 2 Species accumulation curve of endophytic OTUs from Peruvian *Hevea* spp. (*dashed lines*), *Tillandsia* spp. (*dotted lines*) and *Vasconcellea microcarpa* (*solid line*). The vertical dashed line indicates the larges common sample size (26 sequences). Inserted small figure displays species richness of this sample size in dependence of altitude. The regression line and test statistics (see text) indicate non-significance

Fig. 3 ITS-based Bayesian phylogeny of endophytic fungi from *Vasconcellea microcarpa* (*black tips*), *Tillandsia* spp. (*grey tips*) and *Hevea* spp. (*white tips*) rooted with members of Taphrinales. Each terminal branch represents an OTU based on 97 % ITS1 similarity, bar length shows the number of sequences per OTU. *Shortest bars* represent singletons. *Thickened branches* indicate posterior probabilities above 70 %. The identical fully annotated tree is provided as Supplementary material 4

richness of *Tillandsia* endophytes was in the range of *Hevea* endophytes. Plotting species richness in dependence of altitude (insert of Fig. 2) revealed the lowest value (of *Vasconcellea* endophytes) at highest altitude (~3,000 m a.s.l.). Overall correlation of species richness and elevation was not significant (adjusted $R^2 = 0.2$, $p = 0.11$). Species richness estimations remained unstable for all data. That means that none of the estimators' curves reached saturation. Therefore graphics are not shown and the results are not discussed at length but can be rerun from the supplementary R-source file using the indata files provided as Supplementary material 3.

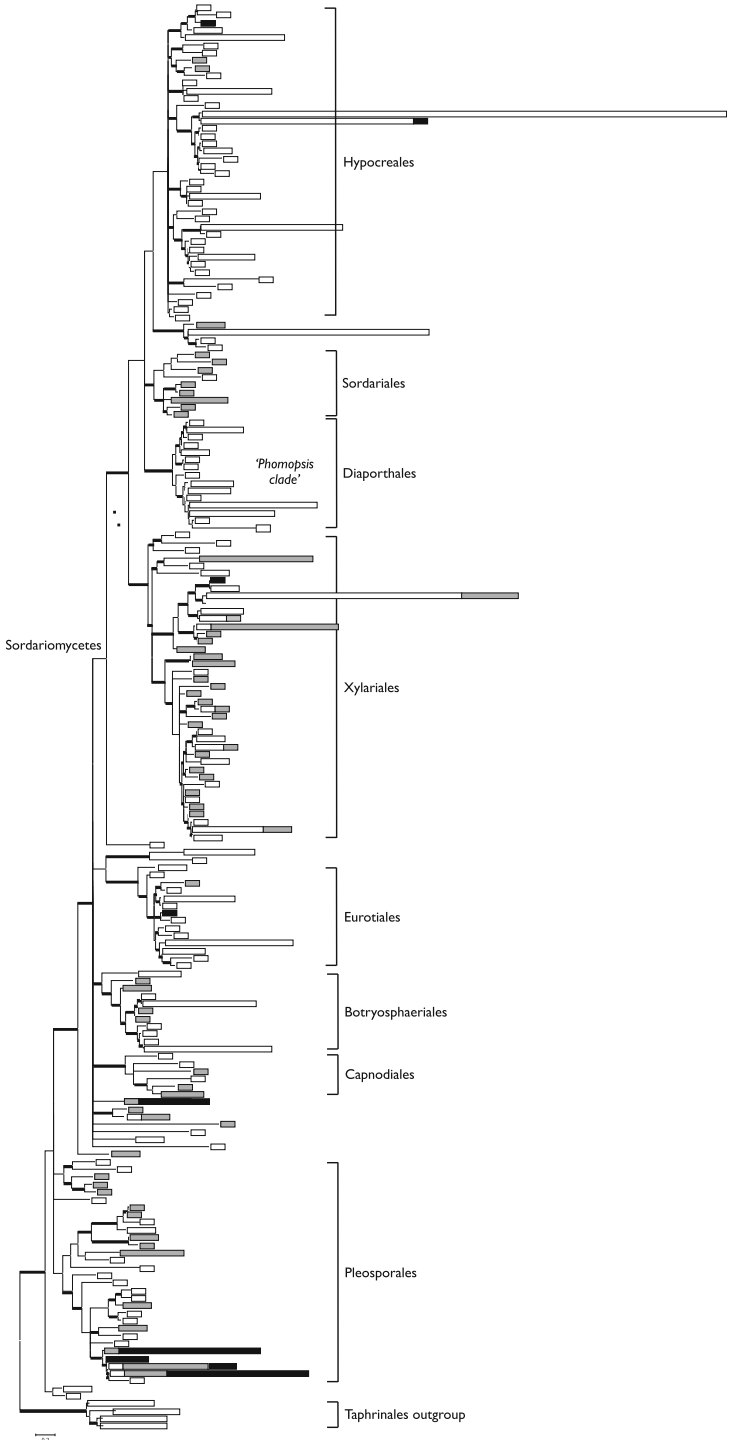
Phylogenetic analysis of fungal endophytes

Bayesian ITS phylogeny revealed known ascomycete orders for the majority of fungal endophytic OTUs (Fig. 3). The figure further displays the plant source of fungal isolates: *V. microcarpa* (black), *Tillandsia* spp. (grey) and *Hevea* spp. (white), whereas the length of the tips indicates the number of sequences per OTU with the shortest bars representing singletons (one sequence per OTU) (an annotated version of this tree is provided as online Supplementary material 4). Endophytes found in this study, belonged to eight major orders of Ascomycota (Basidiomycete endophytes are not considered in this study). The fungal orders Hypocreales (42 OTUs), Xylariales (41 OTUs) and Pleosporales (30 OTUs) exhibited the highest genetic diversity; the Diaporthales were represented by a single and genetically closely related clade of the "*Phomopsis* form group", which were exclusively isolated from *Hevea* spp. This was also the case for OTUs from Hypocreales, whereas Sordariales were represented by OTUs isolated predominantly from *Tillandsia* species. The Xylariales were equally composed of isolates from *Tillandsia* spp. and *Hevea* spp. (Fig. 3). Endophytes from *V. microcarpa* leaves were unequally distributed within the ITS tree. The majority of isolates from highland papaya and 4 out of 9 of OTUs belonged to a closely related group within the Pleosporales with closest BLAST hits for *Phoma/Epicoccum* (Supplementary material 2, "esm02_peru_ascos_main.xls" spreadsheet "OTU_BLAST_hits_rep1").

Community structure

Cluster analysis, non-metric multidimensional scaling (NMDS) and detrended correspondence analysis (DCA not shown) revealed the significant distinctiveness of observed assemblages according to their host plants (Fig. 4a, b; anosim $R = 0.80$, $p = 0.001$). In NMDS, three sampling sites and their corresponding endophytic assemblages were separated from the two groups of Peruvian *H. brasiliensis* and *Tillandsia* spp. endophytes: *T. usneoides* endophytes from Florida, USA (Fig. 4 TILusn.FL) also formed a separate cluster (Fig. 4a).

Hevea-endophytes from Mexico (HEVbra.MX) and Cameroon (HEVbra.CM) exhibited similarities to fungi from Peruvian *Hevea* sites, as shown in the cluster analysis (Fig. 4a). *Vasconcellea* endophytes grouped together with fungi isolated from *Tillandsia* (Fig. 4a). Two further environmental factors, altitude (anosim $R = 0.32$, $p = 0.22$) and geographic position measured as decimal GPS coordinates had no significant effects on species composition (Mantel test $R = 0.009$, $p = 0.49$).


Hypocreales
Sordariales
'Phomopsis clade'
Diaporthales
Sordariomycetes
Xylariales
Eurotiales
Botryosphaeriales
Capnodiales
Pleosporales
Taphrinales outgroup
0.2

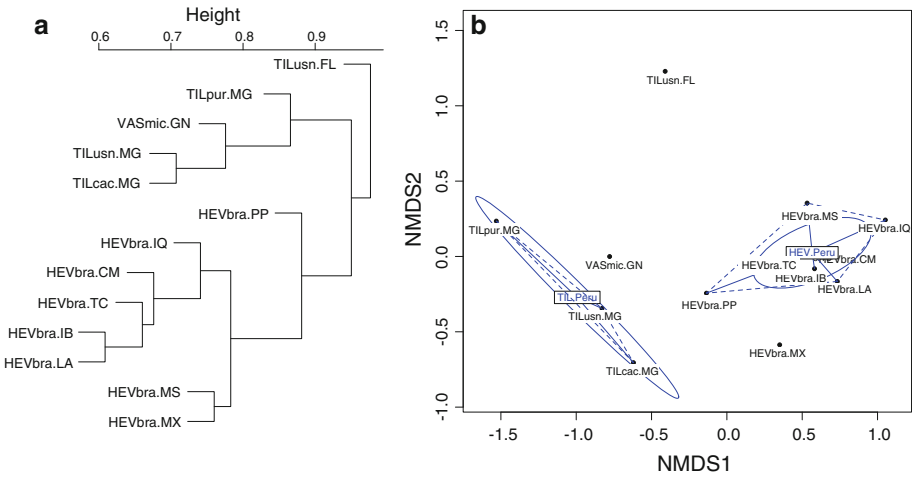


Fig. 4 OTU-dependent average linkage clustering (**a**) and non-metric multidimensional scaling (**b**) of sampling sites. In the NMDS ordination an enclosing hull for all items in a class is drawn (here: host plant genera) and ellipses for class standard errors. Abbreviations contain information about the host plant (*TILusn Tillandsia usneoides, TILpur T.* cf. *purpurea, TILcac T.* cf. *cacticola, VASmic Vasconcellea microcarpa*, *HEVbra Hevea brasiliensis*) and site information (*GN* Granada, *MG* Magdalena, *FL* Florida, *CM* Cameroon, *IB* Iberia, *IQ* Iquitos, *MS* Madre Selva, *MX* Tabasco, *PP* Tambopata, *TC* Tocache, *LA* Los Amigos)

Discussion

The clear under sampling of the underlying fungal communities of Peruvian *Hevea* and *Tillandsia* spp., as indicated by the steeply rising species-accumulation curves (Fig. 2), introduced uncertainty to the richness extrapolation of endophytic species (own data and Coddington et al. 2009; Gazis and Chaverri 2010). In contrast, the endophytic assemblage of *Vasconcellea microcarpa* was clearly poorer in OTU numbers but nevertheless diversity estimators also failed to provide stable results. Notwithstanding, valuable conclusions can be drawn from the results of this species richness analysis. According to the randomized species-accumulation curves (Fig. 2), species richness in *V. microcarpa* would never reach that of *Hevea* and *Tillandsia* endophytes even if sampling efforts are increased. Observed species richness at all lowland and mid-range samples (*Hevea* and *Tillandsia*) clearly exceeded that of the highland site (*Vasconcellea*).

Sampling sites for *V. microcarpa* in the village of Granada, located at around 3,000 m a.s.l. in the northern Peruvian Andes, were characterized by harsher climate and poorer surrounding vegetation (extended pastures and abundant young pine reforestations on sandy soils, personal observations) in comparison to the Peruvian *Tillandsia* sites which were located a lower altitude (~ 1,000 m) and close to the Utcubamba river (surrounded by diverse vegetation). The remaining *Hevea* sites in lowland tropical forest stands and plantations are also characterized by richer surrounding vegetation (Gazis and Chaverri 2010).

Changes in biodiversity of plants and animals caused by changes in altitude are well known (e.g. Colwell et al. 2008). In contrast, little is known about microbial diversity across different elevational regimes. The first to thoroughly address this serious gap in general understanding of biodiversity were Bryant et al. (2008) who found contrasting patterns of microbial and plant diversity patterns along an elevation gradient in the Rocky

Mountains (Colorado, USA). In their study, phylogenetic diversity (Vane-Wright et al. 1991; Faith 1995) of microbes declined monotonically with increasing altitude, whereas plant richness followed a unimodal pattern with a peak in species richness at mid elevations. Bacterial communities had also a tendency to be more phylogenetically clustered than expected by chance at all elevations (Bryant et al. 2008). These findings cannot be strictly transferred to the present study (cf. Fig. 3), probably because endophytes, unlike soil bacteria, are tightly bound to their plant hosts.

The present data structure (e.g. sampling from different host plants and different geographic locations) did not allow us to draw final conclusions about the effect of altitude on endophyte richness (insert of Fig. 2). However, results from Cordier et al. (2012a) show that endophytic species richness and composition varies along elevation gradients in response to ambient temperature regimes (). In their study the authors recognized particular fungal groups that increased in abundance with increasing altitude (e.g. a putative pathogenic *Mycosphaerella* species).

It has been hypothesized that the pathogen-load for mountain vegetation might increase with ongoing global warming (Cordier et al. 2012a). In the present study, such a presumed "pathogen-load" was clearly observed for the highland papaya *V. microcarpa* from which 25 out of 39 isolates belonged to the *Epicoccum*/*Phoma* complex (Fig. 3). This is a group of fungi that contain many plant-pathogens (de Gruyter et al. 2009). However, members of this group can also be neutral or even mutualistic plant invaders (Carroll 1988; Slippers and Wingfield 2007, de Lima Favaro et al. 2011). For instance, *Epicoccum nigrum* is known for its mycoparasitic activity against *Cochliobolus sativus*, a known pathogen of cereals (Campbell 1956). At the time of sampling, all *Vasconcellea* plants looked healthy and even older leaves showed no symptoms of disease. It was not tested here, but the plants might be vaccinated with this endophytic species preventing the attack of pathogenic fungal strains.

Despite the comparatively small subsample of 30 sequences per site out of an exhaustive sequence pool of *Hevea* endophytes (Gazis and Chaverri 2010, Gazis et al. 2011) the present Bayesian ITS phylogeny confirmed original taxonomic range of *Hevea* endophytes (Gazis and Chaverri 2010). The phylogeny further produced evidence for endophyte clades preferring a host plant over the other (Fig. 3). Members of the Diaporthales were not isolated from *V. microcarpa* and *Tillandsia* spp. Instead they belonged to a well-defined *Phomopsis* clade isolated only from *Hevea* spp. Further possibly *Hevea*-selective clades were observed within the Hypocreales (this order was very poorly represented among *V. microcarpa* and *Tillandsia* spp. isolates), the Eurotiales and the Botryosphaeriales. In contrast the Xylariales, Capnodiales and Pleosporales lacked host-selective clades. The Sordariales group contained predominantly isolates from *Tillandsia* spp.

This molecular evidence for host-specific assemblages of fungal endophytes was confirmed by multivariate analysis (Fig. 4). This statistics relied only on the discrimination of OTUs based on a sequence similarity threshold and not on the discrimination between closely and distantly related OTUs. Both cluster analysis (Fig. 4a) and NMDS (Fig. 4b) displayed the sampling sites well separated from each other according to their respective host plants. The most distinctive host plant according to the nature of its endophytic assemblage was *Tillandsia usneoides* from Florida (U'Ren et al. 2012). The remaining *Tillandsia* sites and the *V. microcarpa* site coincided into one group (Fig. 4a), reflecting their geographic proximity (Fig. 1). However, the influence of geographic position on the nature of fungal assemblages (Arnold and Lutzoni 2007, U'Ren et al. 2012) remained

insignificant probably due to the nature of our data or the choice of ITS instead of a multilocus analysis (Gazis et al. 2011; Vrålstad 2012).

Several factors are currently known to influence leaf-inhabiting fungal endophytes: the structure and diversity of surrounding vegetation (Helander et al. 2007), elevation (temperature; Cordier et al. 2012a) and geographical position (U'Ren et al. 2012), urbanness and rurality (Jumpponen and Jones 2010), host plant identity (Unterseher et al. 2007, 2012), population structure of host plant species (Cordier et al. 2012b) and seasonality (Jumpponen and Jones 2010; Schoch et al. 2012). Morphological and physiological peculiarities of host plants should not be disregard. Latex sap (*Hevea*), CAM metabolism (*Tillandsia*) or proteolytic enzymes in *Vasconcellea* could well influence the dynamics, richness, composition Hand host-specificity of their fungal communities. Analysis of these factors; however, were beyond the scope of this study.

Considering the huge diversity of landscapes, habitats and endemic plants in Peru (e.g. Swenson et al. 2012) as well as the matchless importance of fungi in ecosystems (e.g. Molina et al. 2011), perspectives for intensified fungal biodiversity research, in particular in the Peruvian Andes-Amazon region are enormous. Forthcoming intensified studies should deliver baseline data on the state of biodiversity, detect changes in the region, and identify potential threats for biodiversity by the analysis of fungal indicator/umbrella species. Gain of scientific knowledge might then also positively influence regional economic development. Future projects should therefore create opportunities for the training of field assistants (local student helpers, advanced scientists and untrained people) thus strengthening the awareness of intrinsic and economic value of biodiversity (Lovell et al. 2009). Scientific activities, such as the one presented here, enable internationally visible state-of-the-art research at small universities through fair and equitable sharing of resources, data and infrastructure.

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