Leaf-inhabiting fungal endophytes of *F. benjamina, F. elastica, F. religiosa* from Philippine tropical forests and German botanical greenhouses - Assessment of diversity, community composition and bioprospecting perspective

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Ernst-Moritz-Arndt-Universität Greifswald

vorgelegt von

Michael Jay L. Solis

geboren am 30.09.1976

in Bacolod City, Philippines

Dekan: Prof. Dr. Werner Weitschies

Gutachter: PD. Dr. Martin Unterseher

Gutachter: Prof. Dr. Marc Stadler

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PREFACE

This cumulative dissertation is the culmination of many years of mycological interests conceived from both personal and professional experiences dating back from my youthful hobbies of fungal observations and now, a humble aspiration to begin a mycological journey to usher Philippine fungal endophyte ecology forward into present literature. This endeavour begun as a budding mycological idea, and together with the encouraging and insightful contributions from Dr. Martin Unterseher and Dr. Thomas dela Cruz, this has developed into what has become a successful 3-year PhD research work. The years of work efforts included interesting scientific consultations with botanical experts from Leipzig, Bochum, Berlin, Greifswald, Rostock that would lead into the final selection of *Ficus* host species, and eventually in their timely collections. The Philippine aspect of the project also fostered meaningful collaboration with the University of Santo Tomas Research Center for Natural Sciences and the Philippine Department of Natural Resources with valuable exchange of expert knowledge about ideal sites for conducting biodiversity surveys and selecting robust *Ficus* species to represent Philippine natural forest and urban habitats. Towards the end, *Ficus religiosa, Ficus benjamina and Ficus elastica* were chosen as the best candidates and the pursuing independent laboratory work for isolating and collecting fungal endophytes eventually came to fruition.

As an overview, all data and knowledge generated from this research project is presented in the form of mycological papers. In total, 4 papers would sum up this cumulative dissertation, comprising of two publications, one submitted paper and one paper in preparation. Generally, all works dealt primarily with the biodiversity and phylogeny of leaf-inhabiting fungi of three *Ficus* species (*F. benjamina*, *F. elastica* and *F. religiosa*) with the exception of the bioprospecting paper which focused on discovering antimicrobial activities and secondary metabolite production. Investigations took place in natural and urban forests in the Philippines and in tropical greenhouse gardens in Germany.

The first paper (Solis et al. 2015) deals with the basidiomycetous yeasts encountered in surprisingly high numbers in *Ficus* hosts from German greenhouses. The second paper (Solis et al. 2016) focused on fungal endophyte biodiversity and host-related community patterns from the sampled natural forests in the Philippines. The third study covered the overall

comparison of endophyte diversity and community composition (Solis et al., submitted). The final paper deals with the preliminary screening of fungal endophye isolates for their ability to produce antimicrobials and discern which are best candidates for the identification of secondary metabolites (Solis et al., in preparation)

PUBLICATIONS

Accepted Papers

1. Solis MJL, Yurkov A, dela Cruz TE, Unterseher M. 2015. Leaf-inhabiting endophytic yeasts are abundant but unevenly distributed in three *Ficus* species from botanical garden greenhouses in Germany. *Mycological Progress*. 14:1019, http://dx.doi.org/10.1007/s11557-014-1019-6.

2. Solis MJL, dela Cruz TE, Schnittler M, Unterseher M. 2016. The diverse community of leaf-inhabiting fungal endophytes from Philippine natural forests reflects phylogenetic patterns of their host plant species *Ficus benjamina*, *F. elastica* and *F. religiosa*. *Mycoscience*, http://dx.doi.org/10.1016/j.myc.2015.10.002.

Submitted Papers

1. Solis MJL, dela Cruz TE, Unterseher M. Cultivated leaf-inhabiting endophytic fungi from fig tree species (*Ficus* spp.) in German tropical greenhouses form distinct communities compared with natural outdoor conditions of the *Philippines. Biodiversity and Conservation*.

Manuscript in preparation

1. Solis MJL, Merdivan S, Unterseher M, Lindequist U. Preliminary screening for antimicrobial activities of leaf-inhabiting fungal endophytes from fig tree species (*Ficus* spp.).

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1. Abstract

1.1 English

To uncover the biodiversity of leaf-inhabiting fungal endophytes of *Ficus* host species (*F. benjamina*, *F. elastica*, *F. religiosa*) in different natural sites in the Philippines and greenhouses in Germany, asymptomatic leaf samples were collected between November 2012 and March 2013 from five collection sites (Mt. Makiling forest reserve, Mt. Palay-Palay forest reserve, Manila City urban forest, Greifswald University Botanical Garden, Berlin Botanical Garden). Processing of leaf samples using dilution-to extinction culturing to isolate fungal endophytes were used. In total, 450 leaves or leaf units were collected, of which 2,398 fungal isolates were recovered. Representative morphotypes of fungal isolates were selected for sequencing of the internal transcribed spacer region of the rDNA gene (ITS).

From a total of 1,273 fungal isolates recovered from German botanical gardens, 191 isolates emerged as white and pink-pigmented unicellular yeasts (Solis et al. 2015). Following MSP-PCR and ITS1 analysis, yeasts strains were clustered into 23 unique OTUs. Taxonomic annotation (eg. with BLAST) and ITS phylogeny revealed all OTUs separated into two genera: *Cryptococcus* (Filobasidiales; Agaricomycotina; Tremellomycetes) and *Rhodotorula* (Microbotryomycetes and Cystobasidiomycetes; Pucciniomycotina). The yeasts *R. lysiniphila* and *C. albidosimilis* were most abundant, with 89 (47 %) and 43 (23 %) isolates. Species richness estimations predicted between two and six additional yeast species to be isolated with the same methods. The corresponding multivariate analysis revealed clear overlapping of endophyte communities, either based on host or site preferences. Community statistics confirmed this visual impression (ANOSIM of Bray-Curtis dissimilarities for factor site: p=0.32, R=0.15; for factor host: p=0.35, R=0.22). Multivariate analysis did not support host preference, as samples showed no distinct grouping according to host identity or locality.

In both tropical forests in the Philippines, a total of 1,125 isolates were recovered from Mt. Makiling and Mt. Palay-Palay natural forest reserves, of which 400 isolates were selected for ITS sequencing (58% of total isolation effort) (Solis et al. 2016). OTU clustering revealed 54 (97% similarity) and 60 (99% similarity) distinct OTUs. Diversity indicators Shannon, Hill N1 and N2 estimated Mt. Makiling as more diverse than the Mt. Palay-Palay site. Species accumulation curves revealed incomplete sampling, but different curve shapes indicated lowest species richness for *F. religiosa*, intermediate values for *F. benjamina* endophytes and highest values for *F. elastica*. NMDS and PCO ordinations showed overlapping community composition for the two sites. This result was confirmed with ANOSIM (R=- 0.15, p=0.6). High similarity was detected between the fungal assemblages of *F. elastica* and *F. benjamina* (12 shared OTUs), but a distinct composition from *F. religiosa* (3 shared OTUs with both *F. elastica* and *F. benjamina*). ANOSIM confirmed the general significant effect of host identity for fungal community composition (R= 0.98, p › 0.001).

One main result of the third study of the entire dataset certainly is the clearly differing endophyte diversity between the greenhouse and the outdoor samples, with lower estimated total species richness for the first than for the second group (Chao 2: 106 ± 25 OTUs for greenhouse and 253 ± 66 OTUs for the outdoor samples) (Solis et al., submitted). The most diverse order belonged to the Capnodiales with 51 OTUs. Members of the Mycosphaerellaceae were almost exclusively isolated from the natural habitats in the Philippines, whereas OTU richness and abundance of the Davidiellaceae (eg. *Cladosporium*)

were higher in samples from German greenhouses (Solis et al., submitted). NMDS and PCO ordinations showed contrasting community composition between the botanical greenhouses in Germany and the natural habitats/outdoor environments in the Philippines. The endophytic community was significantly related to the type of environment as tested with PERMANOVA ($F = 3.54$, $R2 = 0.22$, $p = 0.001$) whereas the influence of host species in shaping the endophytic communities remained without insufficient statistical support ($F = 1.06$, $R2 = 0.13$, $p = 0.326$). The minor influence of host identity is clearly visible in the ordinations.

In our aim to incorporate an applied dimension to this predominantly pure scientific research, hence to search for biotechnology valuable natural products from our collected isolates, the final work included an exhaustive preliminary search for antimicrobials among candidate fungal endophyte isolates. The highlights included significant and pronounced inhibitions of test bacteria in culture plates induced by 14 fungi from its extracellular and intracellular crude extracts. Notable bioactive species were *Amyloporia* sp. and *Phomospsis* spp. with strongest and broad-spectrum antimirobial activity and thus are recommended for downstream chemical analysis. The remaining 5 bioactive fungal genera (eg. *Acremonium*, *Colletotrichum*, *Mycosphaerella*, *Sardiomycete*, *Talaromyces*) were also significant for bioactivity with specific actions against test bacteria. Here we conclude the presence of antimicrobials from a diversity of fungal endophytes where habitat and hosts are suggested to have an influence in secondary metabolite production.

During the past three years, both positive and negative aspects of our cultivation-based study became clearly visible: On the one hand, cultivation is labour intensive and requires stable infrastructure and similar lab conditions, if sample processing is done at different places, as it was the case here. Due to time and money constraints, many isolates had to be transferred from agar plates to reaction tubes and back to agar plates before and after transport from tropical Manila to Greifswald. Irrespective of appropriate hygenic measures, contamination was inevitable and resulted in a considerable loss of data in the end.
One the other hand, the work with living cultures gave us the unique possibility for bioprospecting. Research on this intensively started in cooperation with the working group of Prof. Lindequist, together with Simon Merdivan and it is our aim that these studies can be continued and would lead to publishable results in the near future.

1.2 Deutsch

Um die Biodiversität von Blatt bewohnenden endophytischen Pilzen an *Ficus* Bäumen (*F. benjamina*, *F. elastica*, *F. religiosa*) zu untersuchen, wurden lebende Blätter an natürlichen Standorten auf den Philippinen sowie in Gewächshäusern der Botanischen Gärten in Greifswald und Berlin zwischen November 2012 und März 2013 gesammelt. Die Blattproben wurden zur Isolation und Kultivierung der Endophyten weiterverarbeitet, indem sie zu Blattpartikeln zerkleinert, filtirert und stark verdünnt wurden. Anschließend erfolgte die Ausplattierung auf ein Komplett-Nährmedium (Malz Extrakt Agar - MEA), von dem entstehende Pilzkolonien auf neue axenische Petrischalen überimpft wurden.

Insgesamt wurden 450 Blätter verarbeitet, aus denen 2398 Pilzkulturen isoliert wurden, davon ca. die Hälfte - 1273 - aus den Gewächshäusern Greifswald und Berlin. Die Pilze wurden in sogenannte Morphogruppen eingeteilt und eine repräsentative Auswahl an Isolaten wurde für die Sequenzierung der sogenannten ITS region weiter verwendet.

Aus Blättern des deutschen Materials wurde eine ungewöhnlich hohe Zahl an pink und weiß gefärbten Hefen isoliert (Solis et al. 2015). Durch die Anwendung einer DNA fingerprinting Methode (MSP-PCR: Microsatellite primed PCR) sowie der ITS sequenzierung wurden die merkmalsarmen Pilze in 23 Gruppen, den sogenannten OTUs (operational taxonomic units) zusammen gefasst. Die taxonomische Zuordnung mittels BLAST und der ITS Sequenz Datenbank UNITE ergab, daß sämtliche OTUs zu den beiden Gattungen *Rhodotorula* (Microbotryomycetes, Basidiomycota) und *Cryptococcus* (Filobasidiales, Tremellomyces, Basidiomycota), gehörten. Die Arten *R. lysiniphila* und *C. albidosimilis* waren die häufigsten Arten mit jeweils 89 bzw. 43 Isolaten (47 % bzw. 23 %).

Eine Analse der Artenzusammensetzung mit multivariaten Methoden ergab eine starke Überlappung sowohl zwischen den Wirtsarten, als auch zwischen den beiden Standorten Greifswald und Berlin. Eine fehlende deutliche Wirts- bzw. Standortpräferenz konnte statistisch abgesichert werden (ANOSIM für Faktor Wirt: $p = 0.32$, $R = 0.15$ und für Faktor Standort: $p = 0.35$, $R = 0.22$).

Von Aufsammlungen der beiden natürlichen philippinischen Standorten Mount Makiling und Mount Palay-Palay konnten 1125 Pilze isoliert werden, von denen 400 sequenziert wurden (Solis et al. 2016). Eine OTU Gruppierung basierend auf 99 % Sequenzähnlichkeit resultierte in 60 OTUs, die Berechnung basierend auf 97 % Sequenzähnlichkeit ergab 54 OTUs. Eine Diversitätsanalyse mit mehreren Indices (Fishers Alpha, Shannon, sowie der ersten beiden Wertender sogenannten Hill-Serie) ergab eine höhere Diversität auf Mt. Makiling als auf Mt. Palay-Palay. Arten-Akkumulationskurven zeigten einen verhältnismäßig geringen Sammelaufwand an, wobei an den Kurvenverläufen deutlich zu erkennen war, daß die höchste Artenvielfalt in Blättern von *F. elastica* und die niedrigste in *F. religiosa* zu erwarten war. Hinsichtlich der Artenzusammensetzung zeigten *F. elastica* und *F. benjamina* eine hohe Übereinstimmung und *F. religiosa* eine eigene Artengemeinschaft. Statistische Tests untertützten die Annahme von klaren Wirtspräferenzen bei einigen der isolierten endophytischen Pilzen ($p > 0.001$, $R = 0.98$).

Als wichtigstes Ergebnis der dritten Studie, in der sämtliche Daten vergleichend analysiert wurden, ist sicherlich die deutliche Abgrenzung der Endophyten Biodiversität aus

Gewächhäusern und natürlichen Standorten. Dies gilt sowohl für die Artenvielfalt wie auch für die Artenzusammensetzung. Schätzungen der Gesamtartenvielfalt ergaben eine deutlich niedrigere Vielfalt in Gewächshäusern (106 ± 25 OTUs) als an natürlichen Standorten (253 ± 66 OTUs). Mehrere OTUs aus der Familie Mycosphaerellaceae (Capnodiales, Ascomycota) wurden ausschließlich aus Blättern von natürlichen Standorten isoliert, wobei Vertreter der nahe verwandten Familie Davidiellaceae (z.B. *Cladosporium*) deutlich häufiger aus den Gewächshausbäumen stammten. Multivariate analysen der Artenzusammensetzung (NMDS - non-metric multidimensional scaling und PCoA - principal components analysis) zeigten die oben erwähnte klare Unterteilung in eine Artengemeinschaft der Gewächshäuser und der natürlichen Standorte, was mit statistischen Tests abgesichert wurde (PERMANOVA: $F = 3.54$, $R^2 = 0.22$, $p = 0.001$). Im Gegensatz zur zweiten Studie, in der ein deutlicher Einfluss der Wirtsart erkennbar war, waren Wirtsart und Artenzusammensetzung der Endophyten nicht mehr signifikant korreliert ($p = 0.326$).

Ein weiteres Projekt im Rahmen der Doktorarbeit war die Erforschung von ausgewählten Pilzarten und Kulturen hinsichtlich ihrer Fähigkeit zu Produktion von biotechnologisch interessanten Inhaltsstoffen. In Kooperation mit Prof. Dr. Ulrike Lindequist und dem Kollegen Simon Merdivan wurde in einem ersten Schritt ein umfangreiches Screening durchgeführt. Dabei wurden ausgewählte Pilze in sogeannten Inhibitionstests mit Bakterien inkubiert und auf ihre Bioaktivität hin untersucht. Vierzehn Pilzarten zeigten deutliche Aktivitäten, wobei neben *Acremonium*, *Colletotrichum*, *Mycosphaerella*, *Sardiomyces* und *Talaromyces* die beidenTaxa *Amyloporia* und *Phomopsis* die deutlichsten und breitesten antibiotischen Reaktionen zeigten und für weitere Tests ausgwählt wurden. Zusammenfassend ist bei dieser letzten, noch laufenden Studie zu erwähnen, daß ein breites Spektrum von kultivierbaren endophytischen Pilzen offenbar deutliche bioaktive Potentiale aufweist und daß möglicherweise sowohl Standort wie auch die Wirtsart einen Einfluss auf die Produktion von pilzlichen sekundären Metaboliten ausüben.

1. INTRODUCTION

Fungal endophytes in tropical forests

Tropical forests are regarded as one of the most species-rich terrestrial ecosystems. In the Philippines, half of ca. 7.2 million ha of forest land are primarily wet tropical forests. With half of the 10,000-12,000 plants as endemic species, 5 percent of the world's flora are found only in the Philippines. However, decades of major habitat destruction with little remaining protected areas, the Philippines has since been identified as among the top conservation priorities in the world. In mycological perspective, the importance of forests cannot be understated simply because a plethora fungal species are associated with plants within forests. Hundreds of fungal species colonize plants that essentially impact plant communities (Toju et al. 2013).

Prior to the development of modern molecular tools, several estimates of the total number of fungi on the basis of their association with plants have been offered (Bass and Richards 2011) Perhaps nothing more recognizable than Hawksworth's 1.5 million species (Hawksworth 1991). However, the rapid development of modern tools and techniques such as large-scale sequencing of environmental samples, as well as data arising from tropical studies will likely modify fungal estimates to a more robust figure. With recent molecular surveys showing thousands of fungal endophyte species harboured by a single plant host (Kemler et al 2013) or scores of species colonizing a single tropical tree leaf (Blackwell 2011), there could be as many as six million species of fungi (Schoch et al. 2014).

The plant-fungi association in natural forests have been generally recognized as a non-random process (Chagnon et al. 2012; Montesinos-Navarro et al. 2012) simply because the formation of fungal endophyte communities are governed by the structuring influences of a wide variety of factors as reported in many fungal surveys. Perhaps nothing more evident is the biogeographic structuring of fungal communities (Meiser et al. 2013). First reported by Arnold and Lutzoni (2007), diversity and incidence of foliar fungal endophytes from diverse plant communities would gradually increase from the North American tundra to lowland tropical forests. This observation was further collaborated by Ikeda et al. (2014) recently where diversity indicators declined from subtropical, cool temperate, to subboreal forests respectively. In addition, endophyte taxonomic composition across decreasing latitudes shifted from a few species of many varying classes to a large number of species of a small number of classes (Arnold & Lutzoni 2007; Hoffman & Arnold 2008). Dispersal limitation

across varying spatial scales may be a key contributor to this pronounced heterogeneity of endophyte assemblages across spatial scales (Higgins et al. 2014).

In forest ecosystems, phylogenetic fungal diversity and endophyte function may be essentially inter-related. In trees, endophyes are likely among the first potential decomposers of dying dead leaves and wood where the flow of carbon, nitrogen and other nutrients is facilitated during the decomposition process. In addition to phylogenetic and taxonomic implications, the production of secondary metabolite production and forest dynamics are also intertwined. Because fungal endophytes have such immense potential to enhance host resistance against herbivores and microbial pathogens through the production of various secondary metabolites (Clay and Schardl 2002), this would potentially impact plant evolution (Brundrett 2006) and biodiversity (Arnold and Lutzoni 2007; Krings et al. 2007).

The fact that tropical forests are such highly regarded host of high fungal diversity (Hawksworth and Rossman 1997; Arnold and Lutzoni 2007), this ecosystem type can be used as a reference to compare with other types (eg. artificial environments, disturbed habitats) that may identify new biodiversity hotspots and set research priorities.

Non-natural habitats of fungal endophytes

In contrast to the natural growth environments of fungal endophytes, non-natural types also pose as interesting but relatively underexplored models for hosting diverse endophyte lineages. In these habitats, biogeochemical cycles are controlled by complex interactions between anthropogenic and environmental factors (Kaye et al. 2006). Such are the cases for artificial wetlands (Cowden and Shefferson 2013), managed plantations (Putra et al. 2015), and domesticated forest areas (Beenhouwer et al. 2014) to name a few examples. Correspondingly, a variety of diversity patterns are observed, often showing a decrease in species richness (Gazis and Charverri 2015) or a transformation of fungal guilds from specialists to generalists (Cowden and Shefferson 2013). Present urban habitats and greenhouse botanical gardens are quite notable for housing a wide array and sometimes unusual range of diverse species.

Urban environments account for only 2% of the earth's land surface (Grimm et al. 2000). In urban habitats, such as city forests, urbanization is the primary driving force which commonly results in landscape fragmentation (McDonnell and Pickett 1990; Ochimura and

Fukuda 2007). Consequently leading to a highly heterogeneous environment and reducing the number of native species (Kowarik 1995), decreasing the biodiversity of trees and herbaceous species (Hattori and Ishida 2000) and fungal species diversity (Bainard et al. 2010).

Foliar fungal endophytes harboured in polluted urban environments are reported to be especially sensitive to sulphuric acid and heavy metal deposition as their numbers is reduced in plants to as much as 80% (Helander et al. 2010). This reduction in endophyte infection was associated with the removal of plant litter due to human land management (Jumpponen and Jones 2010).

Contrastingly to urban habitats, indoor tropical greenhouses are rather pollutant-free. The indoor environment is generally warm, humid and wind-free; conditions which permit not only excellent plant growth, but also bacterial and fungal proliferation, and eventually the development of diseases (Cline et al. 1988). In these facilities, ornamental plants are protected from pathogens and adverse environmental conditions such as low temperature and precipitation (Jarvis 1992).

Greenhouses provide significant impact to species diversity as they pose as favourable habitats for non-native fungal species coming from warmer regions (Nentwig 2007; Desprez-Loustau 2009). Notably, some tropical species have been first introduced into greenhouses, some of them later spreading in the wild, eg. *Leucocoprinus birnbaumii* or *Gymnopus luxurians* (Pidlich-Aigner 2002). In addition, alien species, especially invasive ones, have become a growing problem on a global scale, primarily in relation to nature conservation but also in agriculture, forestry, and fish production (Walther et al. 2009; Keller et al. 2011).

Fungal endophytes: biology and plant hosts

Fungal endophyte form specialised associations with various plant species within multilayered, spatially and temporally diverse plant tissues (Sun and Guo 2012). Reciprocating the favourable microbial habitat plants provide, fungal endophytes increase host resistance to herbivores. However, defensive mutualism appears to be most commonly detected in systemic and vertically transmitted grass endophytes compared to horizontally transmitted tree endophytes. Nevertheless, defensive mutualism provides the best

framework for understanding plant-endophyte interactions in general (Saikkonen et al. 2010).

Many fungal endophytes (eg. *Diaporthe/Phomopsis*) are often reported as plant pathogens from a wide range of hosts (Gomes et al. 2013). Similarly, the vascular plant pathogen *Verticillium* have been isolated from asymptomatic plants. These examples strongly suggest the latent pathogenic phase of fungal endophytes (Porras-Alfaro and Bayman 2011).

Over the many surveys conducted on a wide range of extreme environments, fungal endophytes are omnipresent, isolated as psychrotrophs (Li et al. 2012), xerophiles (Unterseher et al 2012) etc., indicating their high adaptability in extreme conditions.

Endophyte communities are non-random structures (Toju et al. 2013). For example, some taxons are considered as being host specific when it exclusively occurs on the stated host, but not on the other hosts in the same habitat (Holliday 1998). Alternatively, many endophytic fungi occur within more than one host with different percentage occurrence among the hosts in the same habitat. The term "host preference" has been coined for this and indicates a common occurrence or uniqueness of occurrence of a taxon on a particular host or substratum (Zhou and Hyde 2001).

Methodological perspectives

Despite the rapid advancements in environmental sequencing technology, many present fungal surveys are still primarily based on culture-dependent methods and morphological approaches. The fact that these traditional methods can still yield a relatively high proportion of fungi that can be grown in cultures (Sun et al. 2012). Typically, species delimitation is carried out on the basis of morphological variations, so called morphotypes are often used as a proxy for species. However, this method may overestimate diversity and commonly produce large number of non-sporulating endophytic fungi (Lacap et al. 2003) that appear physically indistinguishable (Premalatha and Kalra 2013). Consequently, DNA-based diagnostics have been incorporated to characterize diversity of fungi (Glaeser and Lindner 2011). Here, DNA sequencing of the internal transcribed spacer (ITS) and large subunit (LSU) regions of nuclear ribosomal DNA (rDNA) has proved especially useful for identification (Schoch et al. 2012). Nevertheless, culturing fungi from environmental samples will remain an indispensable step for the purpose of species identification, taxonomic

revisions, assessments of environmental roles, and providing strains for biotechnological experimentations (Nilsson et al. 2006).

Still, cultural techniques are problematic as in the case of typically emerging fast-growing fungi in cultures that are favourably isolated while unculturable and slow-growing fungi remained undetected (Duong et al. 2006). To somehow reduce such limitations, a high-throughput culture method based on the dilution-to-extinction technique (Collado et al. 2007; Unterseher and Schnittler 2010) is suggested. With this method, host leaf explants in the case of foliar fungal endophytes are reduced to micro-particle sizes following standard surface sterilization protocols. This method is based on the principle that one particle would host 1 fungal cell that would consequently allow slow-growing fungi a chance to grow in culture.

In light however of aiming to achieve ecologically meaningful insights into fungal diversity and ecology, next-generation sequencing technologies (NGS) with its capability for deeper and broader taxon sampling and the simultaneous sequencing of millions of DNA fragments (Margulies et al. 2005, Siddique and Unterseher 2016) will become the primary and ideal tool for present and future environmental studies (Tedersoo et al. 2010; Davey et al. 2013).

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This PhD research project has been designed to address the absence of mycological information of tropical endophyte biodiversity, especially in the overwhelmingly underexplored regions of the paleotropics. We therefore aimed to address the main question: Is there high fungal endophyte diversity in tropical *Ficus* species in natural and artificial environments? To answer this question, we specifically seek to answer: 1) Are fungal endophytes communities species rich and phylogenetically diverse? 2) Which host species and environment carry the highest diversity 3) Do fungal communities have similar species composition and structure? 4) Do leaf-inhabiting endophytes in these hosts follow host-specificity?

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3. PUBLICATIONS

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ORIGINAL ARTICLE

Leaf-inhabiting endophytic yeasts are abundant but unevenly distributed in three *Ficus* species from botanical garden greenhouses in Germany

Michael Jay L. Solis · Andrey Yurkov · Thomas Edison dela Cruz · Martin Unterseher

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Abstract Yeasts of both Ascomycota and Basidiomycota occur in various ecological zones of many geographic regions and climatic conditions, but environmental yeast research has often been conducted in either extreme habitats or the phyllosphere. Here, we report on the occurrence of foliar endophytic yeasts of three tropical *Ficus* species from two German greenhouses in Greifswald and Berlin. Living leaves were collected and subjected to dilution-to-extinction cultivation. Fungal colonies were used for morphological analyses, microsatellite-primed fingerprinting, sequencing and phylogeny of the internal transcribed spacer (ITS) DNA. Fifteen percent (~200 colonies) of all fungal isolates belonged to the genera *Cryptococcus* (Filobasidiales) and *Rhodotorula* (Sporidiobolales and Cystobasidiales) that split into 23 species / operational taxonomic units. No other yeast-forming taxa were isolated. Both side-specific and host-specific variations in species composition and abundance were observed; however, statistics did not support significant associations. Further evidence exists that gardening practices, such as moving potted plants, could influence fungal endophytic communities.

Keywords Plant-associated microfungi · Biodiversity · Community ecology · Species richness · Operational taxonomic unit · Leaf latex · Quarantine

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M. J. L. Solis · M. Unterseher (✉)
Ernst-Moritz-Arndt University Greifswald, Institute of Botany and Landscape Ecology, Soldmannstr. 15, 17487 Greifswald, Germany
e-mail: martin.unterseher@uni-greifswald.de

M. J. L. Solis
Department of Natural Sciences, College of Science and Information Technology, Ateneo de Zamboanga University, La Purisima St., 7000 Zamboanga City, Philippines

A. Yurkov
Leibniz-Institut DSMZ-German Collection of Microorganisms and Cell Cultures, Inhoffenstraße 7 B, 38124 Braunschweig, Germany

T. E. dela Cruz
Department of Biological Sciences, College of Science, University of Santo Tomas, Espana, 1015 Manila, Philippines

Introduction

Fungal endophytes live as mutualists or latent pathogens within plant tissues either partly or throughout their life cycle. They cause no apparent disease symptoms (Rudgers et al. 2012; Delaye et al. 2013). Since the recognition of clavicipitaceous fungi as defensive mutualists of host grasses (reviewed in Faeth and Saari 2012; Wali et al. 2013), successive endophyte explorations were undertaken in various environments, i.e. tropical, temperate, arctic or xeric habitats (Suryanarayanan et al. 2011; Unterseher et al. 2012; Bezerra et al. 2013; Matsumura and Fukuda 2013) and from diverse host lineages, i.e., mosses and liverworts (Zhang et al. 2013; Yu et al. 2014), ferns and fern allies (Muthukumar and Prabha 2013), and angiosperms and gymnosperms (Orlandelli et al. 2012; Yoo and Eom 2012; Thongsandee et al. 2012; Lau et al. 2013). Most plants, if not all, are recognized to harbor fungal endophytes (Aly et al. 2011; Porras-Alfaro and Bayman 2011; Dickie et al. 2013), therefore directly impacting plant ecology, survival, fitness and evolution (Brundrett 2006; Woodward et al. 2012; Mayerhofer et al. 2013), as well as community structure (Clay 1999; Worchel et al. 2013; Toju et al. 2013). For example, the production of biologically active secondary metabolites of endophyte-infected hosts (Aly et al. 2010; Tanaka et al. 2012) obviously renders resistance against microbial pathogens (Waller et al. 2005) and the relative success of the host against herbivore attacks (Czarnoleski et al. 2010; Crawford et al. 2010).

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Yeasts, often synonymized with the fermenting ascomycete *Saccharomyces cerevisiae*, are found in all lineages of basidiomycota, i.e., the Pucciniomycotina, Agaricomycotina and Ustilaginomycotina (Kurtzman et al. 2011). They occur in various habitats, some of which are considered to be extreme, such as hydrothermal and deep-sea environments (Gadanho and Sampaio 2005), glacial and subglacial ice (Buzzini et al. 2012), antarctic sea sediments (Zhang et al. 2012), aquatic habitats (Kutty and Philp 2008; Coelho et al. 2010), forests (Maksimova and Chernov 2004; Yurkov et al. 2012b) and swamps (Kachalkin and Yurkov 2012). In extreme environments, they have been found as psychrotolerant (e.g., Buzzini et al. 2012), halotolerant (Butinar et al. 2007), acidophilic (Gross and Robbins 2000; Gadanho and Sampaio 2006) and alkaliphilic strains (Lisichkina et al. 2003). In natural environments, these microfungi thrive in a variety of plant-related substrates, such as surfaces of vascular and non-vascular plants (Fonseca and Inácio 2006; Castanon-Olivares et al. 2007; Li et al. 2010; Brezna et al. 2010; Barata et al. 2012; Kachalkin and Yurkov 2012), fruits (Isaeva et al. 2009; Maksimova et al. 2009; Santo et al. 2012; Vadkertiova et al. 2012), and decaying wood (Peter et al. 2003; Khan et al. 2010). Yeasts have established diverse associations with plants, such as saprobic stages of smuts and jelly fungi, and also as endophytes in meristematic tissues, leaves, stems, flowers, and fruits (Pirttila et al. 2003; Larran et al. 2007; Wang et al. 2008; Xin et al. 2009; Unterseher and Schnittler 2009; Aburein et al. 2012; de Lima et al. 2013). Consequently, their ecological roles have been associated with plant growth processes (Nutaratat et al. 2014), disease development (Gai et al. 2009; Komatsu et al. 2010) and general microbial interactions (Camatti-Sartori et al. 2005). Studies on fungal endophytes have rarely focused on yeasts so far, despite their ability to adapt in such environments, particularly in their sugar-rich fluids. Several fungi known as common endophytes (e.g., *Aureobasidium pullulans*) are able to switch from yeast to filamentous stage, depending on the environment (Slepecky and Starmer 2009).

In the present study, specific interest was given to the question of species richness, host preferences and the community structure of yeast cultures that showed no yeast–hyphae transition during the incubation period, and which were isolated in unusually large numbers along with endophytic stages of various filamentous fungi. It was hypothesised that community structure of those endophytic yeasts at the species level did not differ between the different *Ficus* hosts.

Materials and methods

Host species, study sites and cultivation of fungal endophytes

Ficus species are currently grouped into 15 clades within their family (Ronsted et al. 2008). *Ficus benjamina* and *F. elastica* thereby belong to the *Conosycea* s.l., while *F. religiosa* groups separately under *Uristigma* s.l. Leaf samples of those host plants were collected from botanical garden greenhouses at two German cities, Greifswald and Berlin. Healthy young and mature leaves with no visible morphological abnormalities, e.g., fungal growth, sclerosis, pigmentation loss, or size abnormalities, were collected in November and December 2012. After collection, leaf samples were immediately processed (Greifswald samples), or transported in sterile bags and processed the same day (Berlin samples). Leaves of *Ficus elastica* and *F. religiosa* were cut into sizes approximating that of *F. benjamina*. Twenty-five pieces/leaves of each *Ficus* species were randomly selected and thoroughly hand-rinsed with sterile distilled water to remove any dirt and debris. They were then surface sterilized by submerging into 70 % ethanol for 2 min, sodium hypochloride (1 % active chloride) for 5 min, and 70 % ethanol for 1 min, respectively. Four plugs per leaf with 10 mm diam. were produced with a sterile cork borer under sterile conditions. The 100 leaf plugs per tree species and site were further homogenized according to the dilution-to-extinction protocol for foliar endophytes (Unterseher and Schnittler 2009). Briefly, the material was homogenized for 1 min (15 s low speed, 15 s medium speed and 30 s full speed) in a disinfected blender containing 200 ml sterile water and then filtered through analytical sieves of different mesh sizes (640, 200 and 100 μm). Leaf particles of 100–200 μm size were washed, diluted 1:5 and 1:10, and plated onto Malt Extract Agar-containing, sterile, 48-well multiwell plates (Carl Roth, Karlsruhe, Germany). The 1.5 % MEA was supplemented with Tetracycline (10 mg l^{-1}) and Cyclosporine (10 μl l^{-1}). Multiwell plates were air-dried under a laminar flow to allow evaporation of excess of water, and incubated at room temperature (21–23 °C) and ambient indirect daylight. The plates were examined regularly for 30 days, and emerging fungal colonies were axenically transferred onto fresh MEA plates for morphological analysis and DNA extraction. Permanent cultures were deposited at the DSMZ—German Collection of Microorganisms and Cell Cultures, Braunschweig.

DNA extraction, amplification and sequencing

Genomic DNA was extracted from the axenic isolates using self-made CTAB/chloroform/isoamylic alcohol/isopropanol protocols or the commercially available MasterPure Yeast DNA Purification Kit (epicentre, Madison, U.S.A). Since our yeast strains were almost indistinguishable (except for their pinkish and whitish colouration), PCR fingerprinting (Yurkov et al. 2011) with the microsatellite-specific oligonucleotide $(GAG)_5$ as a single PCR primer was used to group pure cultures. Strains showing identical electrophoretic profiles were considered as conspecific, and only one to five representatives of each group were chosen for further

identification by sequencing of the ITS rDNA region. Amplification of the internal transcribed spacer (ITS) region was performed with primers ITS 1 and ITS 4 (White et al. 1990) under standard concentrations and conditions on an Eppendorf Mastercycler 15.64 μl ddH_20, 5 μl Mango-Buffer, 1.7 μl $MgCl_2$ (Bioline, 50 mM), 0.5 μl dNTP (10 mM), 0.5 μl of each primer (10 pM/ μl), 0.16 μl (5 U/ μl) of Taq DNA polymerase and 1 μl template DNA; 5 min at 94 °C, followed by 35 cycles (35 s at 94 °C, 50 s at 52 °C, 1 min 30 s at 72 °C), and 5 min at 72 °C. PCR products were run on agarose gel (0.8 %) for control and were shipped to Beckman Coulter Genomics (Takeley, England) for sequencing. Chromatograms were manually inspected to eliminate sequencing errors. Forward and reverse sequences were aligned and remnants of the flanking 18S and 28S rDNA were removed with ITSx (Bengtsson-Palme et al. 2013). INSD accession numbers and other basic data are provided below and as online supporting information.

OTU delimitation

Pairwise similarities among ITS1 sequences were calculated using Local BLAST (ftp://ftp.ncbi.nih.gov/blast/executables/release/2.2.9/; last accessed June 2014) with the parameters '-m8 -r2 -G5 -E2'. The R function 'simMatrix' from package RFLPtools (Persoh et al. 2010) was applied to transform the calculated pairwise similarities into a similarity matrix, and a hierarchical cluster analysis (R function 'hclust', R Development Core Team 2012) was conducted to group similar ITS1 genotypes to OTUs by the 'average linkage' method and a threshold of 99–95 % sequence similarity. A rough preliminary BLAST search was performed to identify and separate ascomycete and basidiomycete yeasts.

Alignment, phylogenetic analysis and taxonomy

In addition to our own sequences (all were basidiomycota and based on the 99 % OTU threshold), sequences from INSD covering the whole phylogenetic range of basidio-yeasts were included in the present ITS phylogeny. Sequences were "ITS-extracted" (Bengtsson-Palme et al. 2013) and aligned with MAFFT version 6 using the E-INS-i strategy (Katoh and Toh 2008) with slight manual refinement using Mesquite version 2.75 (Maddison and Maddison 2011). Phylogenetic analysis combined maximum likelihood (ML) and Bayesian interference (BI). ML was run on raxmlGUI (Silvestro and Michalak 2012) using the rapid bootstrap option with 1,000 replicates. For Bayesian analysis, the appropriate model for minimum evolution was selected from the 24 models implemented in MrModeltest 2.1 (Nylander 2004). 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 5 Mio., 1000 and 0.25, respectively. Clade support was assessed with posterior probabilities. Sequences were deposited under accessions LK022803 to LK022840. In deciding about the most appropriate OTU threshold for taxonomic assignment of the yeasts, we evaluated OTU grouping (95–99 %), ITS phylogeny (with 99 % OTUs), compared the results with public databases (e.g., CBS), and added our own expertise.

Community analysis

Species-sample data were compiled from the OTU/species table considering both incidence (presence/absence) and abundance information of a species in a given sample. Species richness and community composition (i.e., host preferences) were analysed with R (Team 2012). R commands and input data are provided as online supporting information. Species richness analysis for OTUs used mathematically smoothed (i.e., randomized) species accumulation curves to display the accumulation of "species" when the number of records increased (Gotelli and Colwell 2001). By analysing the shape of the curves (e.g., initial slope, approaching an asymptote or not), it was possible to evaluate basic patterns of species richness (observed species richness, sampling depth). In addition, species richness estimators Chao, Jackknife 1, and Bootstrap previously applied for fungi (Unterseher et al. 2008, 2013b) and yeasts (Yurkov et al. 2011) were calculated. Non-metric multidimensional scaling (NMS or NMDS) based on Bray-Curtis dissimilarities was used to visually assess the influence of environmental variables (here "host" and "site") of fungal OTUs.

Results

Species richness, taxonomy and phylogenetic placement

Fifteen percent of all isolates (191 strains) grew as stable, pinkish or whitish yeasts without transformation into a filamentous stage. Such yeast-mycelium transitions were observed for other isolates during this study, e.g., for the ascomycete *Aureobasidium pullulans*. MSP-PCR analysis of the yeast isolates resulted in 37 different electrophoretic patterns. In total, 119 strains were sequenced. OTU clustering of the sequences based on the ITS1 region revealed 13 (95 % similarity threshold), 23 (97 %) and 38 OTUs (99 %). Further downstream analyses (identification with OTU clustering, MSP-PCR, BLAST and ITS phylogeny) showed that the 97 % OTU grouping was the most reasonable compromise of the four approaches, and it was finally chosen as the basis for species lists and taxonomic annotations of representative

Table 1 Species list and meta data of endophytic yeasts from *Ficus* spp. Columns "Strain No." and "Accession" indicate the identity of representative cultures deposited at the DSMZ, Braunschweig, and the GenBank identifier of the corresponding ITS sequences

Site	Host	Taxon	No. of isolates	Strain no.	Accession
Berlin	*Ficus benjamina*	*Rhodotorula mucilaginosa*	6	1269	LK022803
		Cryptococcus albidosimilis	5	1272	LK022804
		Rhodotorula slooffiae	1	1224	LK022805
	Ficus elastica	*Rhodotorula lysiniphila OTU 1,2*	6	1068 1087	LK022806 LK022807
		Cryptococcus albidosimilis OTU 1, 2	5	1148 1131	LK022808 LK022809
		Rhodotorula mucilaginosa	2	1115	LK022810
		Rhodotorula minuta	1	1114	LK022811
	Ficus religiosa	*Cryptococcus albidosimilis*	1	1000	LK022812
		Rhodotorula mucilaginosa	1	971	LK022813
Greifswald	*Ficus benjamina*	*Rhodotorula lysiniphila OTU 1–3*	72	572 744 655	LK022814 LK022815 LK022816
		Cryptococcus albidosimilis OTU 1–3	26	803 734 779	LK022817 LK022818 LK022819
		Rhodotorula slooffiae	12	566	LK022820
		Rhodotorula minuta	7	866	LK022821
		Rhodotorula mucilaginosa	7	580	LK022822
		Cryptococcus magnus OTU 1, 2	5	814 683	LK022823 LK022824
		Cryptococcus albidus OTU 1, 2	2	777 753	LK022825 LK022826
		Rhodotorula benthica	1	613	LK022827
	Ficus elastica	*Rhodotorula lysiniphila OTU 1, 2*	11	377 276	LK022828 LK022829
		Rhodotorula mucilaginosa	3	257	LK022830
		Cryptococcus magnus	2	351	LK022831
		Cryptococcus albidosimilis	1	304	LK022832
		Cryptococcus albidus	1	263	LK022833
		Cryptococcus stepposus	1	509	LK022834
		Rhodotorula benthica	1	326	LK022835
	Ficus religiosa	*Cryptococcus albidosimilis*	5	105	LK022836
		Cryptococcus magnus	2	124	LK022837
		Rhodotorula mucilaginosa	2	101	LK022838
		Cryptococcus albidus	1	201	LK022839
		Cryptococcus diffluens	1	234	LK022840

GenBank sequences (Table 1). All yeasts from this study separated into the two genera: *Cryptococcus* (Agaricomycotina, Tremellomycetes, Filobasidiales) and *Rhodotorula* (Pucciniomycotina, Microbotryomycetes and Cystobasidiomycetes). The yeasts *R. lysiniphila* and *C. albidosimilis* were most abundant, with 89 (47 %) and 43 (23 %) isolates. *R. mucilaginosa* and *R. slooffiae* followed with 21 and 13 isolates, respectively. All remaining yeast species (*C. albidus, C. diffluens, C. magnus, C. stepposus, R. benthica* and *R. minuta*) had less than nine isolates each (Table 1). No yeasts producing ballistosconidia were isolated. Species accumulation curves for the Greifswald and Berlin isolates and for both data sets are shown in Fig. 1. Species richness estimations predicted between two and six additional yeast species to be isolated with the same methods (estimator curves not shown; however, supplementary R script explains how to calculate them).

Phylogenetic analysis of yeast endophytes from Ficus

Figure 2 displays the phylogenetic placement of *Ficus* yeast sequences within the major yeast-containing basidiomycete groups. Sequences from our own *Cryptococcus* isolates were placed in the Filobasidiales lineage of Tremellomycetes, and *Rhodotorula* isolates were distributed between the two clades, Sporidiobolales (*R. mucilaginosa*) and Cystobasidiales (*R. minuta*, *R. slooffiae*, *R. benthica*, *R. lysiniphila*).

Community analysis

The most species-rich yeast communities were detected from host trees in the botanical garden of Greifswald. Leaf samples from *Ficus benjamina* and *F. elastica* yielded 144 and 34 isolates comprising eight and seven species, respectively (Table 1). In both hosts, *R. lysiniphia* was the predominant

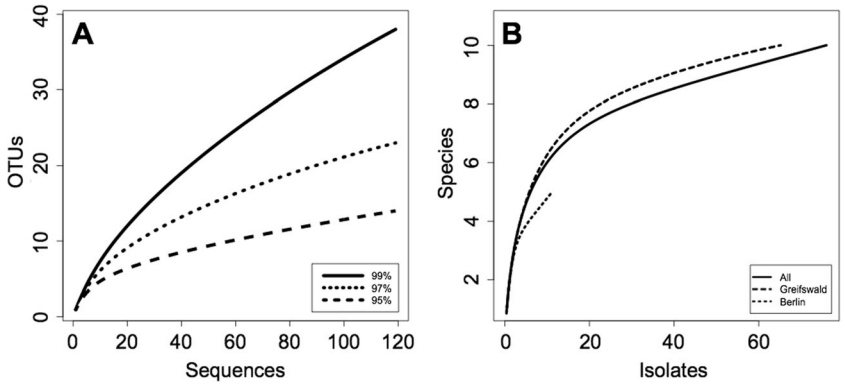

Fig. 1 Species accumulation curves of endophytic yeasts from *Ficus* spp. **a** displays the impact of different OTU thresholds on observed richness, with the number of OTUs plotted against the number of ITS sequences. **b** displays observed species richness of both sites (cf Table 1), with the number of species plotted against the number of isolates. Inserts explain curve signatures

species. *Ficus religiosa* harboured five yeast species with the least number of isolates (13). The three *Ficus* trees from Greifswald shared four yeast species (i.e., *C. albidus, C. albidosimilis, C. magnus* and *R. mucilaginosa*). *Ficus* trees from Berlin harboured five species, all of which were also found among the ten species recovered from Greifswald *Ficus* ssp. The corresponding multivariate analysis revealed clear overlapping of endophyte communities, either based on host (Fig. 3a) or site preferences (Fig. 3b). Community statistics confirmed this visual impression (ANOSIM of Bray-Curtis dissimilarities for factor site: p=0.32, R=0.15; for factor host: p=0.35, R=0.22). Three more yeast species (*C. diffluens, C. stepposus* and *R. slooffiae*) were exclusively isolated from *F. benjamina, F. religiosa* and *F. elastica* respectively; however, in numbers too low to postulate significant host preferences.

Discussion

Diversity and distribution of yeasts on different plant materials have been explored in many studies (e.g., reviewed in Fonseca and Inácio 2006). Additionally, they are known from studies of leaf-inhabiting microfungi, where both ascomycete (e.g., *Aureobasidium*) and basidiomycete yeasts (*Cryptococcus, Sporobolomyces* and *Rhodotorula*) are inherent elements of cultivation-based and environmental sequence data sets (Cordier et al. 2012; Martinson et al. 2012; Unterseher et al. 2013a). In the present study, persistent yeast stages, i.e., strains that did not change to filamentous growth during cultivation, were counted in unusually large numbers from greenhouse plants of one of the most diverse tropical trees—the fig tree (*Ficus* spp.). A total of 191 white and pink yeast strains were obtained from surface-sterilized leaves of *F. religiosa*, *F. elastica* and *F. benjamina* growing in the botanical gardens Berlin and Greifswald.

Species richness of the three *Ficus* hosts

Extensive analysis of the two most common rDNA gene regions (D1/D2 domains of the LSU and the ITS) performed across basidiomycetous yeasts showed distinct sequence heterogeneity patterns in different phylogenetic clades (Fell et al. 2000; Scorzetti et al. 2002). However, in Filobasidiales (except for *Aerius* clade), Sporidiobolales and Cystobasidiales rDNA ITS were generally found to be more variable than LSU. In the present study, ITS1 sequences revealed between 13 (95 % sequence similarity threshold) and 38 (99 %) distinct OTUs. Our multi-way identification separated the many strains into only two genera, *Cryptococcus* and *Rhodotorula*, and showed that the 97 % OTU grouping was the most reasonable compromise of the four approaches. For example, *R. lysiniphila*, *R. benthica* and *R. slooffiae* grouped into a unique OTU at 95 %, whereas at 99 % single species separated too often into different OTUs. Therefore, the 97 % clustering approach was finally chosen as the basis for species lists and taxonomic annotations of representative GenBank sequences, representing ten known taxa (Table 1). Even though the level of intraspecific variability in many different species remains unknown, it has been shown that *R. mucilaginosa* strains show as many as one to four substitutions in the rDNA ITS (Libkind et al. 2008).

Species accumulation curves failed to reach saturation and displayed undersampling for the Berlin data set in particular. However, the prediction of total species richness of *Ficus* leaf endophytes from greenhouses in the Greifswald botanical garden with four estimator functions indicated that the majority of yeast species had been recovered (estimator curves not shown, supplementary R script explains how to calculate them). Such predictions can be used only for the applied method and do not present a working hypothesis about total species richness if other culture conditions, such as lower temperatures or other growth media, would be used. It also does not allow us to predict the number of yeast taxa found with cultivation-independent approaches.


iso 1137
iso 1288
iso 833
Cryptococcus albidosimilis iso 1272 LK022804
iso 750
Cryptococcus albidosimilis iso 1148 LK022808
Cryptococcus albidosimilis iso 1131 LK022809
Cryptococcus albidosimilis iso 1000 LK022812
Cryptococcus albidosimilis iso 105 LK022836
AF444375 Cryptococcus diffluens
AF145325 Cryptococcus albidosimilis
iso 1141
Cryptococcus diffluens iso 234 LK022840
Cryptococcus albidosimilis iso 304 LK022832
iso 845
iso 905
Cryptococcus albidosimillis iso 734 LK022818
AF444412 Cryptococcus vishniacii
AF145327 Cryptococcus kuetzingii
AF444370 Cryptococcus albidus
Cryptococcus albidus iso 777 LK022825
Cryptococcus albidus iso 201 LK022839
Cryptococcus albidus iso 263 LK022833
AF145317 Cryptococcus bhutanensis
AF145326 Cryptococcus antarcticus
AF145322 Cryptococcus friedmannii
AF444372 Cryptococcus saitoi
AF444362 Cryptococcus arrabidensis
AF444350 Cryptococcus terricolus
AF444376 Cryptococcus aerius
NR077081 Cryptococcus fuscescens
AF145318 Cryptococcus elinovii
AF444319 Cryptococcus terreus
AF444383 Cryptococcus wieringae
AF444302 Filobasidium uniguttulatum
Cryptococcus magnus iso 351 LK022831
Cryptococcus magnus iso 124 LK022837
Cryptococcus magnus iso 814 LK022823
AF444450 Cryptococcus magnus
AF190007 Filobasidium floriforme
AF190006 Filobasidium elegans
AF444349 Cryptococcus oeirensis
Cryptococcus stepposus iso 509 LK022834
AF444336 Filobasidium globisporum
AF444304 Cryptococcus gastricus
Filobasidiales
Tremellomycetes
EU149786 Cryptococcus carnescens
GQ911538 Cryptococcus tephrensis
AY040656 Cryptococcus victoriae
AB035047 Cryptococcus peneaus
JN019831 Cryptococcus amylolentus
AF444326 Filobasidiella neoformans
GU585748 Cryptococcus heveanensis
AF314987 Bullera armeniaca
AF444305 Cryptococcus skinneri
Trichosporon clade 1
DQ667177 Cryptococcus humicola
AF444482 Trichosporon ignicola var undulatum
AF444451 Trichosporon moniliiforme
AF444458 Cryptococcus curvatus
Trichosporon clade 2
AF444409 Bullera miyagiana
Tremella clade
AF444338 Cryptococcus flavus
AF444321 Cryptococcus podzolicus
AF444315 Tremella aurantia
AF444399 Bullera pseudoalba
AB035045 Cryptococcus aureus
EF056308 Cryptococcus flavescens
Tremellales and Trichosporonales
Udeniomyces clade
AF444322 Cryptococcus huempii
DQ661022 Xanthophyllomyces dendrorhous
AF144483 Mrakia frigida
AF410469 Cryptococcus aquaticus
AF444418 Guehomyces pullulans
AF444300 Cystofilobasidium capitatum
AF444299 Cystofilobasidium bisporidii
EU082232 Cryptococcus macerans
Cystofilobasidiales
AF444505 Rhodotorula graminis
AF444542 Rhodosporidium babjevae
AF444661 Rhodosporidium diobovatum
AF444510 Rhodotorula araucariae
AF444520 Rhodosporidium kratochvilovae
AF444651 Rhodosporidium toruloides
AF444659 Rhodosporidium sphaerocarpum
AF444660 Rhodotorula mucilaginosa
Rhodotorula mucilaginosa iso 101 LK022838
Rhodotorula mucilaginosa iso 1269 LK022803
Sporidiobolus and Sporobolomyces clade
AF444493 Rhodosporidium paludigenum
Rhodosporidium and Sporidiobolus clade
AF444524 Rhodotorula hordea
AF444532 Rhodotorula javanica
AF444628 Rhodotorula yarrowii
AF444533 Rhodotorula diffluens
AF444641 Rhodotorula nothofagi
AF444574 Rhodotorula fujisanensis
AF444496 Leucosporidium scotti
AF444601 Rhodotorula sonckii
AF444529 Leucosporidium antarcticum
AF444598 Rhodotorula pilati
Microbotryomycetes
AF444494 Kurtzmanomyces nectairei
AF444516 Bensingtonia miscanthi
AF444550 Sporobolomyces ruber
AF444551 Sterigmatomyces elviae
AF444516 Bensingtonia miscanthi
AF444582 Sporobolomyces linderae
Agaricostilbum, Agaricostilbomycetes
AF444597 Sakaguchia dacryoidea
AF444540 Rhodotorula lactosa
AF444522 Erythrobasidium hasegawianum
AF444561 Sporobolomyces elongatus
Sporobolomyces clade
AF444504 Rhodotorula marina
AF444578 Sporobolomyces gracilis
AF444515 Sporobolomyces phyllomatis
AF444523 Rhodotorula armeniaca
AF444544 Sporobolomyces kluyveri-nielii
AF444511 Sporobolomyces salicinus
AF444538 Rhodotorula aurantiaca
AF444567 Occultifur externus
AF444620 Rhodotorula minuta
AF190010 Rhodotorula minuta
Rhodotorula minuta iso 1114 LK022811
AF444627 Rhodotorula slooffiae
Rhodotorula slooffiae iso 1224 LK022805
EU149811 Rhodotorula laryngis
AF444590 Rhodotorula pallida
Rhodotorula benthica iso 326 LK022835
Rhodotorula lysiniphila iso 276 LK022829
Rhodotorula lysiniphila iso 744 LK022815
iso 589
Rhodotorula lysiniphila iso 655 LK022816
iso 910
Rhodotorula lysiniphila iso 572 LK022814
iso 396
Rhodotorula lysiniphila iso 377 LK022828
Rhodotorula lysiniphila iso 1087 LK 022807
Rhodotorula lysiniphila iso 1068 LK022806
Rhodotorula mucilaginosa iso 1069 LK022803
Cystobasidiomycetes
Entyloma clade
AY387150 Malassezia slooffiae
Pseudozyma clade
0.2

◀ **Fig. 2** Majority consensus tree based on Bayesian and maximum likelihood analysis (ML) of basidiomycete yeast ITS sequences. Reference sequences were compiled from Fell et al. (2000) and BLAST searches. Branch support is given as posterior probabilities from Baysian analysis, and if available, as bootstrap support from ML

Species composition and the influence of host and locality

Multivariate analysis did not support host preference, as samples showed no distinct grouping according to host identity or locality (Fig. 3). At this point, it has to be noted that the choice of OTU clustering threshold (97–99 %) did not influence NMDS analysis of community composition in terms of species and sample positions in the two-dimensional ordination space (Fig. 3). Such obvious robustness of this multivariate approach against data manipulation might therefore be suited for molecular data in particular, e.g., from NGS metabarcoding studies.

The obvious absence of host-specific yeast species may be attributed to the ecology of isolated taxa. Most species have been reported from various plant species, including non-vascular plants (e.g., Kachalkin and Yurkov 2012), but also from plant-free sources (e.g., Nagahama et al. 2003; Gadanho and Sampaio 2005; Raggi et al. 2014). It was suggested that yeasts in general could live as unspecialized endophytes with no close associations to their host organisms (Isaeva et al. 2009). The authors further argued that they rely on readily available nutrients (e.g., simple sugars, amino acids, sugar alcohols), thus the yeasts develop rapidly in nutrient-rich environments including plant sap. However, this is mostly applied to ascomycetous yeasts, while basidiomycetes often possess abilities to degrade complex organic compounds (e.g., Sampaio 1999), thus potentially being able to switch from copiotrophic to a decomposer lifestyle. In that same study, phylloplane and endophytic yeast species isolated from succulent fruits were observed to be identical, suggesting that yeast cells transmitted onto fruit surfaces invaded internal tissues by penetrating across the thin fruit covering. Remarkably, wild yeasts populations may successfully survive on host plants over centuries, as it has been shown for the *Saccharomyces* – oak system (Zhang et al. 2010).

In our study, the high number of recovered yeasts could possibly be associated with the nutrient content of the *Ficus* leaf latex. The high calorific value of the hydrocarbon, oil, and protein constituents of the *Ficus* leaf latex could offer a suitable environment for the growth and multiplication of copiotrophic yeasts (Augustus and Seiler 2011; Ogunwande et al. 2011).

On a higher taxonomical level, we find three groups (Cystobasidiales, Sporidiobolales and Filobasidiales) strongly dominating in the endophytic communities (Table 1, Fig. 2). Interestingly, red yeasts members of Cystobasidiales are closely related to the mycoparasite *Cystobasidium fimetarium* (Sampaio and Oberwinkler 2011) and members of Sporidiobolales bear colacosomes (Bauer et al. 2006), which implies their parasitic origin or at least suggests their ability to switch from copiotrophic lifestyle. Slight but insignificant differences in species composition were observed between Greifswald and Berlin samples. These differences became more distinctive when the strictly OTU-based data sets (97 %) were compared instead (not shown). For example, identical OTUs identified as *C. albidosimilis* and *R. lysiniphila* never occurred in samples from both sites. Although it might be highly speculative at the moment, it

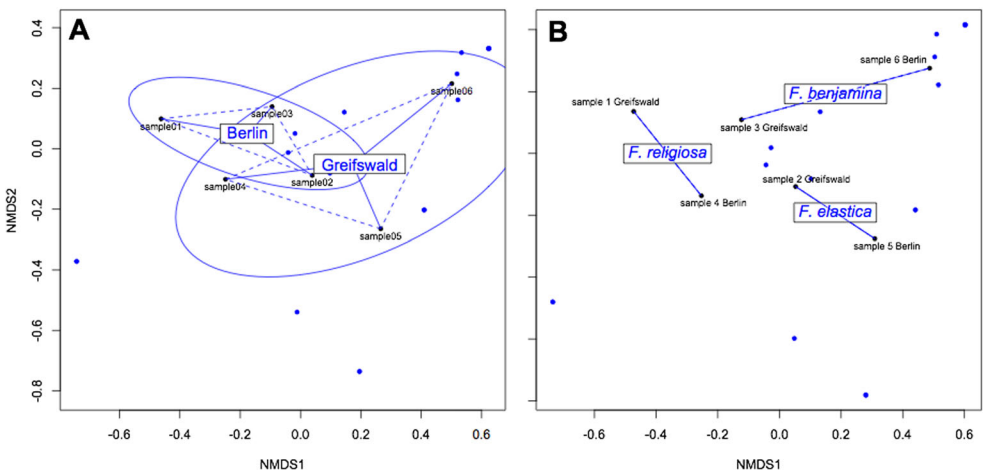

Fig. 3 Results from non-metric multidimensional scaling of species occurrences overlaid with site **a** and host **b** information. Unlabeled dots represent the position of yeast species in ordination space. Please note for (**b**) that although the two corresponding samples for each host are connected, samples from other hosts are located closer to the reference sample, thus contradicting the first impression of host preference of the yeast community, e.g., *F. religiosa* "sample 4 Berlin" is closer to *F. elastica* "sample 2 Greifswald" and *F. benjamina* "sample 3 Greifswald" than to the other *F. religiosa* sample. That means that samples 2, 3 and 4 from three different *Ficus* hosts shared more yeast species than sample 1 and 4 from the same host

could well be that the relevant resolution for niche specialization processes among endophytic yeast might lie below the species level. Furthermore, various environmental factors (pH, nutrients, temperature, humidity, solar radiation, plant exudates, rainfall, seasonal variations, etc.) are often believed to explain differences in yeast abundance and species composition in their natural habitats (Glushakova and Chernov 2004; Vishniac 2006; Nix-Stohr et al. 2008; Kachalkin and Yurkov 2012; Yurkov et al. 2012a, b; Turchetti et al. 2013).

Given that greenhouse systems are man-made, artificial environments, the observed patterns could also be discussed in light of gardening practices and the trees' history. All sampled *Ficus* trees in Berlin have been permanently ground-rooted individuals for more than two decades (pers. communications with gardeners). In contrast, *F. benjamina* and *F. elastica* in the Greifswald Botanical gardens are potted specimens experiencing regular rearrangements within the greenhouse, or outdoors during summer. This results in the exposure of the trees to a broader environment, thus a broader fungal inoculum originating either from air (Frohlich-Nowoisky et al. 2009) or surrounding plants (Allen et al. 2006). These aspects might explain the highest species richness and broadest species composition of *F. benjamina* and *F. elastica* from Greifswald, and point to the importance of quarantine measures to prevent the import of potentially detrimental pathogens that might occur as endophytic blind passengers in living plant material or plant products (Udayanga et al. 2011).

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The diverse community of leaf-inhabiting fungal endophytes from Philippine natural forests reflects phylogenetic patterns of their host plant species *Ficus benjamina*, *F. elastica* and *F. religiosa*

Michael Jay L. Solis [a,b,*], *Thomas Edison Dela Cruz* [c], *Martin Schnittler* [a], *Martin Unterseher* [a]

[a] *Ernst-Moritz-Arndt University Greifswald, Institute of Botany and Landscape Ecology, Soldmannstr. 15, 17487 Greifswald, Germany*

[b] *Department of Natural Sciences, College of Science and Information Technology, Ateneo de Zamboanga University, La Purisima St. 7000, Zamboanga City, Philippines*

[c] *Department of Biological Sciences, College of Science, University of Santo Tomas, España 1015, Manila, Philippines*

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ABSTRACT

Leaf-inhabiting endophytes belong to a diverse and active group of plant-associated fungi harboured in plant-rich tropical environments. Employing dilution-to-extinction cultivation and ITS sequencing, we assessed species richness, phylogeny and community composition of fungal endophytes within healthy leaves of three *Ficus* tree species (*F. religiosa*, *F. benjamina*, and *F. elastica*) naturally growing in the two Philippine forest reserves Mt. Makiling in Laguna and Mt. Palay–Palay in Cavite. Apart from a few basidiomycetes (3 orders, 6 genera), fungal isolates were abundantly ascomycetes (11 orders, 16 genera) and predominated by commonly known endophytic genera, such as *Pseudocercospora*, *Phyllosticta*, or *Penicillium*. Phylogenetic analysis revealed Capnodiales and Eurotiales as most OTU-rich clades and suggesting a high potential pathogen load in the investigated trees. Biodiversity analyses further revealed a higher similarity between the fungal species composition in the leaves of *F. benjamina* and *F. elastica* than to the one in *F. religiosa*. The observed higher abundance, species richness and similarity of the fungal community assemblage in the closely related host species *F. benjamina* and *F. elastica*, suggests an effect of host identity in structuring fungal endophytes community in the tropics.

* *Corresponding author*. Ernst-Moritz-Arndt University Greifswald, Institute of Botany and Landscape Ecology, Soldmannstr. 15, 17487 Greifswald, Germany. Tel.: +49 (0)3834/864184; fax: +49 (0)3834/864114.

E-mail address: mjhay_2000ph@yahoo.com (M.J.L. Solis).

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1. Introduction

Endophytic fungi are a diverse, polyphyletic group of primarily ascomycete species living within asymptomatic tissues of all known plant lineages in a broad range of natural and anthropogenic communities (Bazzicalupo et al. 2013; Matsumura and Fukuda 2013; Chen et al. 2015; Sato et al. 2015) either as permanent or temporary endophytic residents (Márquez et al. 2012; Hodgson et al. 2014). In the latter, fungi may switch between endophytic and necrotrophic lifestyles (Delaye et al. 2013; Stergiopoulos and Gordon 2014) or later on persist as saprobes (e.g., xylariaceous species) during leaf litter decomposition while demonstrating the capacity to utilize various carbon sources (i.e., lignin, cellulose) (Osono 2006; Osono et al. 2013). Although the extent and scale of their ecological roles across biogeographical environments are not fully known (Wani et al. 2015), most studies have synonymized fungal endophytes as primarily mutualists conferring various benefits to its host (e.g., resistance to microbial pathogens, deterrence of herbivore attacks, increase fitness against stressful environments, enhanced physiology and biomass production) (Yuan et al. 2010; Saikkonen et al. 2013).

Ikeda et al. (2014) recently reported the increases of fungal endophyte diversity across a latitudinal gradient from boreal to subtropical forests in Asia. Such a trend was previously observed along the boreal-tropical latitudes of the neotropics (Arnold and Lutzoni 2007). Surveyed neotropical forests revealed the hyperdiversity of fungal endophytes (Arnold et al. 2000, 2001, Arnold and Herre 2003; Gamboa and Bayman 2001; Arnold and Lutzoni 2007), however paleotropic forests showed contrasting diversity (Fröhlich and Hyde 1999; Suryanarayanan et al. 2002; Murali et al. 2007). Tropical fungal endophytic communities typically list a few but frequently-occurring host generalists while the majority as rare specialists (Gamboa and Bayman 2001; Cannon and Simmons 2002; Arnold and Lutzoni 2007; Suryanarayanan et al. 2011). Poor host density in tropical forests accounts for the large disproportion between fungal specialists and host species (May, 1991). Consequently, host-specificity in investigated tropical fungal guilds are generally absent (Murali et al. 2007; Parfitt et al. 2010; Tedersoo et al. 2010). Often, abiotic factors (e.g., geographic distance, climate, seasonal and spatial variations, microclimates, disturbances) are described as drivers of fungal diversity in tropical environments. For example, dissimilarity among endophyte community assemblages depended on the forest types (Suryanarayanan et al. 2011) and temperature and rainfall (Zimmerman and Vitousek 2012). Endophyte abundance and leaf fragment preferences were influenced by elevation, temperature and precipitation (Vaz et al. 2014b), while distribution patterns over local (0–100 km) and regional scales (101–5000 km) were due to geographic distance and environmental factors respectively (Vaz et al. 2014a).

The Philippine archipelago is among the most important repositories of global biodiversity with a very high concentration of endemic plant species (Myers et al. 2000). In the face of potentially novel plant-associated fungal communities and patterns of diversity persisting in these underexplored regions of the paleotropics, the knowledge pertaining to forest species diversity is virtually unknown. In addition, the high abundance of plant species typically found in forests makes host selection an important criteria for testing diversity hypothesis (i.e., host influence, site influence). Fig trees are among the largest, globally-distributed group of plants (Berg 2003). Among the hundreds of *Ficus* species, three species (*F. religiosa*, *F. elastica*, *F. benjamina*) display multiple common attributes: high distribution and abundance in local forests, relevant roles in forest ecology, high tolerance and adaptability to stress conditions, growth in several types of environments (e.g., forests, indoor environments, highly urbanized habitats, tropical greenhouses), global distribution, and phylogenetic positions within the *Ficus* genera to assess host phylogeny/identity influences on plant-associated fungal communities. To date, leaf-inhabiting fungal endophytes are unexplored in these host species. This study was conducted to elucidate the community, including diversity and richness composition of leaf-inhabiting endophytes of native *Ficus* tree species from tropical areas in the Philippines. Among the available hosts, three species were chosen to contain a pair of the closely related taxa *F. benjamina* and *F. elastica* (both sect. *Conosycea*, Rønsted et al. 2005) and a more distantly related one (*F. religiosa*, sect. *Urostigma*, Cruaud et al. 2012). Specifically, we seek to shed light on the fundamental aspect of fungal host preferences on the community level, or, in other words we want to address the following questions: does host identity influence species diversity and community composition of leaf-inhabiting fungal endophytes?

2. Materials and methods

2.1. Host species

The fig trees (*Ficus* spp.) belong to a comparatively large plant genus of predominantly tropical distribution. Its ca. 750 species have been grouped into six subgenera (i.e., *Ficus*, *Urostigma*, *Pharmacosycea*, *Sycomorus*, *Sycidium* and *Synoecia* (Berg 2003)). Within the subgenera *Urostigma*, *F. benjamina* and *F. elastica* belong to subsection *Conosycea* while *F. religiosa* grouped separately under subsection *Urostigma* (Rønsted et al. 2008; Cruaud et al. 2012). The three *Ficus* hosts (i.e., *F. benjamina*, *F. elastica*, *F. religiosa*) are naturally distributed within mostly tropical and a few subtropical Asia–Pacific regions (e.g., Nepal, Bhutan, India, Indonesia, China, Philippines, in other parts of Southeast Asia, North Australia, South Pacific Islands). These plant species preferably grow either on loam or sand soil but vary under varying soil pH. *Ficus elastica* adapts well to broad range tolerances (acidic to alkaline soil), however *F. benjamina* and *F. elastica* prefer slightly acidic to slightly alkaline and neutral to highly alkaline soil respectively. *Ficus* leaves are distinctly evergreen; however habit and morphology (i.e., height and leaf dimensions) differ among species. *Ficus elastica* (thick, oblong leaves, 35 × 17 cm) and *F. religiosa* (broadly ovate to ovateorbiculate leaves, 7 × 4 cm) typically grow up to 30 m high while *F. benjamina* (oblong, elliptic, lanceolate, or ovate leaves, 4 × 1.5 cm) reach heights of only 10 m. Male and female flowers of all *Ficus* host species occur separately (monoecious) in individual trees.

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2.2. Study sites

The collection sites are located in the main Luzon Island of the Philippine Archipelago (Fig. 1): Mt. Makiling (eastern slope); Los Baños, Laguna; and Mt. Palay–Palay/Mataas na Gulod National Park, Ternate, Cavite. Mount Makiling (4224 ha, elevation 1109 m) and Mt. Palay–Palay/Mataas na Gulod National Park (4000 ha, elevation 648 m), comprising primary and secondary forests with a tropical monsoon climate. Mount Makiling stands 65 km south of Manila covered with distinct vegetations between high (900–1109 m above sea level) and low elevations (100–900 m above sea level); the former comprised of mossy forest zones with rugged and steep terrain predominantly covered with narra (*Pterocarpus indicus*) and mahogany (*Swietenia macrophylla*) while the latter with dipterocarp and grassland zones composed mainly of hardwood species and forest plantations respectively. The forest reserve presents a mean monthly temperatures of 23.8 °C–30.4 °C, short dry months (Jan–Apr) and long wet months (May–Dec), annual mean precipitation of 1972 mm. Mount Palay–Palay/Mataas na Gulod National Park is located 60 km southeast of Manila and 60 km northwest of Mt. Makiling. The topography varies from low, moderate, to steep terrains with forest floor and low altitude elevations. Vegetation in low elevation is covered with grasses and herbs which gradually transitions into dipterocarp trees, cogon grasses (*Imperata cylindrica*), shrubs, herbs, vines and ferns at higher elevations. Monthly temperature ranges from 19 °C to 28 °C, with dry months falling between Nov to Apr and wet months May to Oct, and annual mean precipitation of 2000 mm.

2.3. Field work

Collections were made within an elevation rage of 50–150 m asl covering an area of approximately 25 sq km in Mt. Makiling (14.136389 N 121.194444 E) and Mt. Palay–Palay (14.233311 N 120.654502 E). Healthy and mature leaves (largest leaves in an adult tree ca. 10–15 m high) with no visible morphological abnormalities (i.e., fungal growth, sclerosis, pigmentation loss, size abnormalities) were collected randomly from (3–5) individual trees between Feb and Apr 2013. After collection, leaf samples were transported in sterile bags and processed within 24 h. Under sterile conditions, the larger leaves of *F. elastica* and *F. religiosa* were cut into uniform sizes approximating the leaf length of *F. benjamina* (5 in). Each cut represented one leaf. Twenty-five leaves of each *Ficus* species were randomly selected and thoroughly hand-rinsed with sterile distilled water to remove any dirt and debris. Washed leaves were then surface sterilized by submerging into 70% ethanol for 2 min, sodium hypochloride (1% active chloride) for 5 min, and 70% ethanol for 1 min respectively. Four plugs per leaf with 10 mm diam were produced with a sterile cork borer under sterile conditions. The 100 leaf plugs per tree species and site were further homogenized according to the dilution-to-extinction protocol for foliar endophytes (Unterseher and Schnittler 2009). In total, 600 leaf plugs were used (25 leaves × 4 plugs × 3 *Ficus* species × study sites). Briefly, the material was homogenized for 1 min (15 s low speed, 15 s medium speed and 30 s full speed) in a disinfected blender containing 200 ml sterile water and then filtered through analytical sieves of different mesh size (640, 200 and 100 μm). Leaf particles of 100–200 μm size were washed, diluted 1:5 and 1:10 and plated onto sterile 48-well multiwell plates (Carl Roth, Karlsruhe, Germany) pre-filled with Malt Extract Agar. The 1.5% MEA was supplemented with Tetracycline (10 mg l^{-1}) and Cyclosporine (10 μl l^{-1}). Multiwell plates were air-dried under a laminar flow to allow evaporation of excess of water and incubated at room temperature (23–25 °C) and ambient indirect daylight. The plates were examined regularly for 30 d and emerging fungal colonies were axenically transferred onto fresh MEA plates for morphological analysis and morphotype grouping. Mycelium pieces of culture duplicates were prepared for overseas shipping to Greifswald to continue cultivation and DNA extraction.

2.4. DNA extraction, amplification and sequencing

Fungal genomic DNA was extracted from the axenic tissue using common CTAB/Chloroform/Isoamylic alcohol/Isopropanol protocols or the commercially available MasterPure

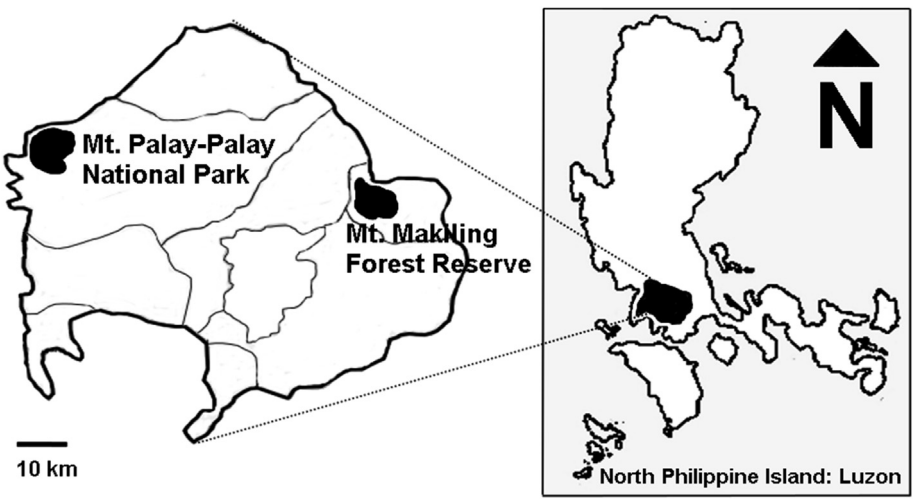

Fig. 1 – Map showing the 2 collection sites (Mt. Palay–Palay National Park and Mt. Makiling Forest Reserve) located in Southern Luzon Island.

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Yeast DNA Purification Kit (epicentre, Madison, U.S.A). Amplification of the internal transcribed spacer (ITS) region was performed with primers ITS 1 and ITS 4 (White et al. 1990) on an Eppendorf Mastercycler (15.64 μl ddH_2O, 5 μl Mango-Buffer, 1.7 μl $MgCl_2$ (Bioline, 50 mM), 0.5 μl dNTP (10 mM), 0.5 μl of each primer (10 pM/μl), 0.16 μl (5 U/μl) of Taq DNA polymerase and 1 μl template DNA); 5 min at 94 °C, followed by 35 cycles (35 s at 94 °C, 50 s at 52 °C, 1 min 30 s at 72 °C), and 5 min at 72 °C. PCR products were run on agarose gel (0.8%) for control. The unpurified amplicons were shipped to Beckman Coulter Genomics (Takeley, England) for sequencing. Chromatograms were manually inspected to eliminate erroneous sequences. Forward and reverse sequences were aligned and remnants of the flanking 18S and 28S ribosomal RNA gene were removed with ITSx (Bengtsson-Palme et al. 2013). Sequences were deposited under accessions LN865089–LN865142. INSD accession numbers and other basic data are provided below and as online supporting information.

2.5. *Morphotype grouping and OTU delimitation*

Pairwise similarities among ITS1 sequences were calculated using Local BLAST 2.2.9 with the parameters '-m8 -r2 -G5 -E2'. The ITS1 was used the DNA barcode for OTU delimitation due to higher sequences variability and higher efficiency for species discrimination than ITS 2 (Wang et al. 2015). The R function 'simMatrix' from package RFLPtools (Persoh et al. 2010) was applied to transform the calculated pairwise similarities into a similarity matrix, and a hierarchical cluster analysis (R function 'hclust', R Development Core Team 2012) was conducted to group similar ITS1 genotypes to OTUs by the method 'average linkage' and a threshold of 99–95% sequence similarity. In order to decide about the most appropriate OTU threshold for taxonomic assignment of the filamentous fungi, we compared the different OTU groupings with morphotype information, ITS phylogeny (with 99% OTUs) and BLAST searches.

2.6. *Phylogenetic analysis and taxonomy*

Both in-house and GenBank sequences were aligned with MAFFT 6 using the E-INS-i strategy (Katoh and Toh 2008) with slight manual refinement in Mesquite version 2.75 (Maddison and Maddison 2011). Phylogenetic reconstruction was conducted using Maximum Likelihood (ML) and Bayesian Interference (BI). ML was run on raxmlGUI (Silvestro and Michalak 2012) using the rapid bootstrap option with 1000 replicates. For Bayesian analysis the appropriate model for minimum evolution was selected from the 24 models implemented in MrModeltest 2.1 (Nylander 2004). 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 5 Mio., 1000 and 0.25 respectively. Clade support was assessed with posterior probabilities.

2.7. *Species richness, diversity indicators and community analysis*

Abundance-based species data were used for analysis of species richness and further diversity indices as well as community composition. Richness patterns were analysed with Fisher's alpha, Shannon index and the first three Hill numbers from Hill's series of diversity (H0 = species richness, H1 = exponent of Shannon index and H2 = inverse Simpson index; Hill 1973). Only if a community or assemblage of species display consistently higher values across all indicators can it be considered as truly more diverse than another species set.

Species richness analysis for OTUs further used mathematically smoothed (i.e., randomized) species accumulation curves to display the accumulation of "species" when the number of records increases (Gotelli and Colwell 2001). By analysing the shape of the curves (e.g., initial slope, approaching an asymptote or not), it was possible to evaluate basic patterns of species richness (observed species richness, sampling depth). Non-metric multidimensional scaling (NMDS) and principal coordinate analysis (PCO; PCoA) based on Bray–Curtis dissimilarities of OTU abundances were used to visualize community patterns. The distinctiveness of endophyte assemblages of the two sites and the three host species *F. benjamina*, *F. elastica* and *F. religiosa* was tested with an analysis of similarities (anosim). For the host data, we increased the power of statistics by creating five random subsamples for each host category. Anosim was then performed with 10000 permutations and visualized with Box–Whisker plots (see Supplementary R-script for the corresponding implementation in R).

Since all steps of biodiversity analysis were conducted with the statistical environment "R" (R Development Core Team), the corresponding script is available as Supplementary material.

3. Results

3.1. *Species richness and further diversity indicators*

Healthy leaves of *Ficus* hosts assessed for the presence of fungal endophytes produced growths of both fertile (spore-forming) and sterile mycelia. In total, 694 isolates were recovered from the two collection sites (i.e., Mt. Makiling and Mt. Palay–Palay natural forest reserves). An accurate morphotype grouping based on colony characters was problematic due to too many similar looking strains. In this study, we defined morphotypes as isolates with similar morphological traits such as colony colour, colony margin, colony shape, spore size, spore shape, presence of growth rings, and growth patterns. Morphotypes with 10 or less isolates were all included in ITS sequencing while only 10 isolates were selected for morphotypes with more than 10 isolates. At the end, 400 isolates for ITS sequencing (58% of total isolation effort) were used for sequencing, from which 364 high-quality ITS sequences were obtained and used to re-assign all other morphotype strains into specific OTU groups. OTU clustering

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based on the ITS1 region revealed 51 (95% similarity), 54 (97% similarity) and 60 (99% similarity) distinct OTUs. The intermediate similarity threshold represented the most appropriate compromise between morphology, BLAST analysis and phylogeny (see below) and was used further to build species lists (Table 1) and OTU tables.

With the exception of Fisher's Alpha (and Hill N0), which revealed comparable values for the two sites, the remaining

Table 1 – List of OTUs obtained after clustering at 97% similarity and molecular identification using BLAST and UNITE including meta data of endophytic fungi from *Ficus* spp.; columns "Strain no." and "Accession no." indicate the identity of representative cultures and the GenBank identifier of the corresponding ITS sequences; Fr – *Ficus religiosa*, Fe – *F. elastica*, Fb – *F. benjamina* refer to the host species.

OTU	Taxon	Family	Order	Host	Collection site	Strain no.	Accession no.
32	Agaricomycetes sp.	–	–	Fb	Makiling	1587	LN865106
43	*Aspergillus* sp. 1	Aspergillaceae	Eurotiales	Fe, Fb	Palay–Palay	1825a	LN865116
46	*Aspergillus* sp. 2	Aspergillaceae	Eurotiales	Fe	Palay–Palay	2042c	LN865118
53	*Aspergillus* sp. 3	Aspergillaceae	Eurotiales	Fe	Palay–Palay	2220	LN865123
70	*Aspergillus* sp. 4	Aspergillaceae	Eurotiales	Fe	Makiling	1693	LN865137
59	Basidiomycota sp.	–	–	Fr	Makiling	2500	LN865129
3	*Cladosporium* sp.	Cladosporiaceae	Capnodiales	Fr	Palay–Palay, Makiling	2057	LN865089
20	*Colletotrichum* sp.	Glomerellaceae	Glomerellales	Fe, Fb	Palay–Palay, Makiling	1578	LN865098
39	*Coniochaeta* sp.	Coniochaetaceae	Coniochaetales	Fe	Makiling	1720	LN865113
69	*Debaryomyces* sp.	Saccharomycetaceae	Saccharomycetales	Fe	Makiling	1647	LN865136
16	*Diaporthe* sp. 1	Diaporthaceae	Diaporthales	Fb	Makiling	1577	LN865094
22	*Diaporthe* sp. 2	Diaporthaceae	Diaporthales	Fb	Palay–Palay, Makiling	1513	LN865099
28	*Diaporthe* sp. 3	Diaporthaceae	Diaporthales	Fb	Makiling	1557	LN865104
38	*Diaporthe* sp. 4	Diaporthaceae	Diaporthales	Fe	Makiling	1712	LN865112
66	*Epicoccum* sp.	Pleosporaceae	Pleosporales	Fe	Makiling	1622	LN865134
37	*Eutypa* sp.	Diatrypaceae	Xylariales	Fe	Makiling	1698	LN865111
35	*Exophiala* sp. 1	Herpotrichiellaceae	Chaetothyriales	Fb	Makiling	1600	LN865109
68	*Exophiala* sp. 2	Herpotrichiellaceae	Chaetothyriales	Fb	Makiling	1604	LN865135
71	*Guehomyces* sp.	Cystofilobasidiaceae	Cystofilobasidiales	Fe	Makiling	1702	LN865138
41	*Ischnoderma* sp.	Polyporaceae	Polypolares	Fb	Palay–Palay	1741	LN865115
61	*Leptosphaerulina* sp.	Didymellaceae	Pleosporales	Fr	Makiling	2502	LN865131
7	*Meyerozyma* sp.	Debaryomycetaceae	Saccharomycetales	Fb	Palay–Palay	1734	LN865091
11	*Mycosphaerella* sp. 1	Mycosphaerellaceae	Capnodiales	Fe, Fb	Palay–Palay, Makiling	1605	LN865093
18	*Mycosphaerella* sp. 2	Mycosphaerellaceae	Capnodiales	Fb	Makiling	1497	LN865096
77	*Mycosphaerella* sp. 3	Mycosphaerellaceae	Capnodiales	Fr	Palay–Palay	2600	LN865142
19	Mycosphaerellaceae sp. 1	Mycosphaerellaceae	Capnodiales	Fe, Fb	Palay–Palay, Makiling	1615	LN865097
23	Mycosphaerellaceae sp. 2	Mycosphaerellaceae	Capnodiales	Fe, Fb	Makiling	1731	LN865100
25	Mycosphaerellaceae sp. 3	Mycosphaerellaceae	Capnodiales	Fe, Fb	Palay–Palay, Makiling	1528	LN865102
49	Mycosphaerellaceae sp. 4	Mycosphaerellaceae	Capnodiales	Fe	Palay–Palay	2146b	LN865121
72	*Penicillium* sp. 1	Aspergillaceae	Eurotiales	Fb	Palay–Palay	1805	LN865139
76	*Penicillium* sp. 2	Aspergillaceae	Eurotiales	Fr	Makiling	2433	LN865141
34	Pezizomycotina sp.	–	–	Fe, Fb	Palay–Palay, Makiling	1593a	LN865108
17	*Phyllosticta* sp. 1	Botryosphaeriaceae	Botryosphaeriales	Fr, Fe, Fb	Palay–Palay, Makiling	2213	LN865095
33	*Phyllosticta* sp. 2	Botryosphaeriaceae	Botryosphaeriales	Fe, Fb	Palay–Palay, Makiling	1592	LN865107
47	*Phyllosticta* sp. 3	Botryosphaeriaceae	Botryosphaeriales	Fe	Palay–Palay	2084	LN865119
60	Pleosporales sp.	–	Pleosporales	Fr	Makiling	2501	LN865130
24	Polyporales sp.	–	Polyporales	Fe, Fb	Palay–Palay, Makiling	1518	LN865101
5	*Pseudocercospora* sp. 1	Mycosphaerellaceae	Capnodiales	Fe, Fb	Palay–Palay, Makiling	1696	LN865090
10	*Pseudocercospora* sp. 2	Mycosphaerellaceae	Capnodiales	Fr, Fe, Fb	Palay–Palay, Makiling	2249	LN865092
26	*Pseudocercospora* sp. 3	Mycosphaerellaceae	Capnodiales	Fe, Fb	Palay–Palay	2085	LN865103
30	*Pseudocercospora* sp. 4	Mycosphaerellaceae	Capnodiales	Fb	Makiling	1582	LN865105
36	*Pseudocercospora* sp. 5	Mycosphaerellaceae	Capnodiales	Fe	Makiling	1633	LN865110
40	*Pseudocercospora* sp. 6	Mycosphaerellaceae	Capnodiales	Fe	Makiling	1723	LN865114
48	*Pseudocercospora* sp. 7	Mycosphaerellaceae	Capnodiales	Fe	Palay–Palay	2106	LN865120
50	*Pseudocercospora* sp. 8	Mycosphaerellaceae	Capnodiales	Fe	Palay–Palay	2148	LN865122
54	*Pseudocercospora* sp. 9	Mycosphaerellacea	Capnodiales	Fe	Palay–Palay	2223b	LN865124
55	*Pseudocercospora* sp. 10	Mycosphaerellaceae	Capnodiales	Fe	Palay–Palay	2231	LN865125
56	*Pseudocercospora* sp. 11	Mycosphaerellaceae	Capnodiales	Fe	Palay–Palay	2248	LN865126
57	*Pseudocercospora* sp. 12	Mycosphaerellaceae	Capnodiales	Fe	Palay–Palay	2257	LN865127
62	*Pseudozyma* sp.	Ustilaginaceae	Ustilaginales	Fr	Makiling	2504	LN865132
58	Sordariomycetes sp.	–	–	Fr, Fe, Fb	Palay–Palay, Makiling	2266c	LN865128
45	*Togninia* sp.	Calosphaeriaceae	Calosphaeriales	Fe	Palay–Palay	2035	LN865117
74	Udeniomyces sp.	Cystofilobasidiaceae	Cystofilobasidiales	Fe	Palay–Palay	2239	LN865140
64	*Ustilago* sp.	Ustilaginaceae	Ustilaginales	Fr	Palay–Palay, Makiling	2508	LN865133

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three diversity indicators Shannon, Hill N1 and N2 estimated Mt. Makiling as more diverse than the Mt. Palay–Palay site (Fig. 2 top row). Comparative diversity analysis based on host identity revealed *F. elastica* as the host species that harboured a higher number of fungal species, while *F. religiosa* hosted the least diverse fungal assemblage (Fig. 2 bottom row). *Ficus benjamina*, which appeared in second place in terms of its endophytic diversity, presented a fungal community more similar to the one present in *F. elastica*. Species accumulation curves revealed incomplete sampling, but confirmed the above mentioned diversity trends with lowest species richness and a flat curve shape for *F. religiosa*, intermediate values and a steeper curve for *F. benjamina* endophytes and highest values for *F. elastica* (Fig. 3). The site-specific species richness of Mt. Makiling and Mt. Palay–Palay was comparable (Fig. 2 – Alpha and Hill N0 and Fig. 3).

3.2. *Phylogenetic analysis and taxonomy of fungal endophytes*

The 54 OTUs clustered under the 97% sequence similarity threshold were distributed within Ascomycota (47 OTUs) and a few Basidiomycota (7 OTUs; Supplementary 02). Well-supported Ascomycota clades (≥70% bootstrap) formed

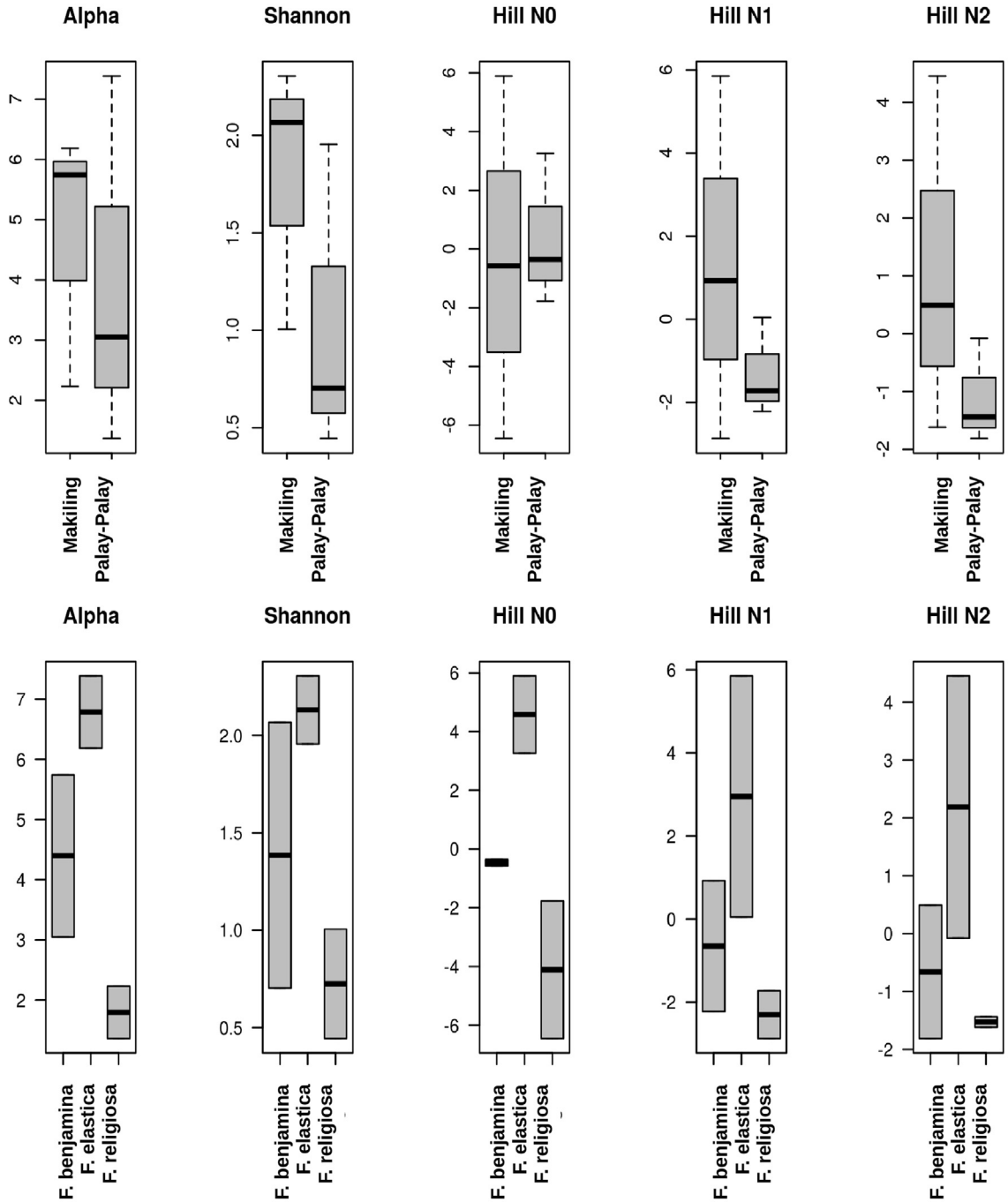

Fig. 2 – Five different diversity indicators showing the impact of location (top row) and of host species (bottom row) on the fungal endophytes from *Ficus* leaves.

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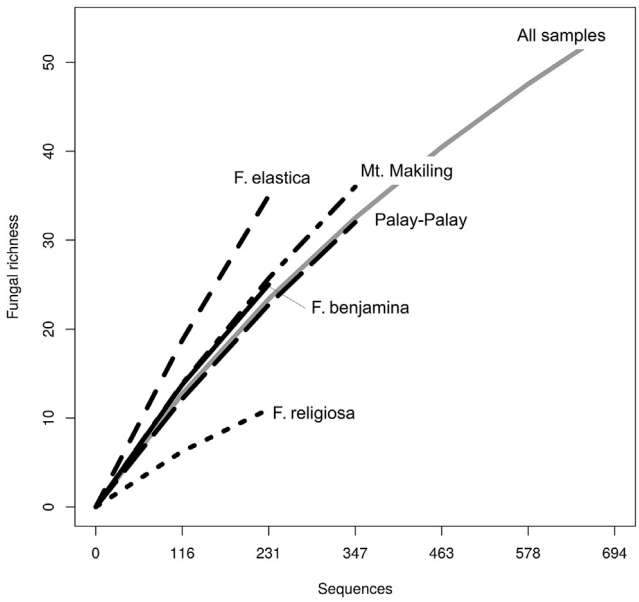

Fig. 3 – Species accumulation curves of fungal endophytes showing the number of OTUs plotted against the number of isolates based on the *Ficus* hosts (*F. elastica*, *F. benjamina*, and *F. religiosa*), the collection sites (Mt. Makiling and Mt. Palay–Palay forest reserves), and all samples pooled from all hosts and collection sites.

eleven orders (Botryosphaeriales, Calosphaeriales, Capnodiales, Chaetothyriales, Coniochaetales, Diaporthales, Eurotiales, Glomerellales, Pleosporales, Saccharomycetales and Xylariales) while Basidiomycota grouped into 3 orders (Cystofilobasidiales, Polyporales and Ustilaginales). The most diverse orders were Capnodiales (20 OTUs, with the majority of OTUs belonging to the *Pseudocercospora*-related species) and Eurotiales (6 OTUs).

Most parsimonious OTU identification (the combination of phylogenetic placement and BLAST searches) led to the annotation of 22 genera, 19 families and 12 orders (Table 1).

3.3. *Community analysis*

Ordinations from both NMDS and PCO showed overlapping community composition for the two sites (Fig. 4A, B). This result was confirmed with anosim (Fig. 4C, $R = -0.15$, $p = 0.6$). Pronounced similarity was also detected between the fungal assemblages of *F. elastica* and *F. benjamina* (12 shared OTUs), but a distinct composition from *F. religiosa* (3 shared OTUs with both *F. elastica* and *F. benjamina*) (Fig. 4D). Anosim further confirmed the general significant effect of host identity for fungal community composition ($R = 0.98$, $p < 0.001$).

4. Discussion

4.1. *Species richness and diversity indicators*

Many biodiversity studies traditionally include species richness analysis in the form of statistically smoothed species accumulation curves (Zhao et al. 2010; Izsak and Papp 2011; Yurkov et al. 2011). Such curves depict sampling effort as well as behaviour of species richness with increasing sample size (Gotelli and Colwell 2001; Moreno and Halffter 2001; Ugland et al. 2003) thus giving valuable information of observed species richness. The present results clearly show that species richness of *F. religiosa* was lowest, with a clear tendency to remain lowest if sample size would increase. For the two other host species, highest endophyte richness cannot be determined with certainty, as it cannot be ruled out that their corresponding species accumulation curves would intersect with increased sample size.

Species richness is but one of several indicators to quantify diversity. For the present analyses, we assessed the behaviour of additional diversity indices, namely Fisher's Alpha, Shannon and the three Hill numbers H0 (similar to richness), H1 (similar to the exponent of Shannon diversity) and H2 (similar to the inverse Simpson index). Fisher's alpha and H0 confirmed results from species accumulation curves, that is a strongly overlapping richness for the two sampling sites and clear differences for the host species, with *F. religiosa* possessing the lowest and *F. elastica* the highest endophyte richness (Fig. 2). The three additional indicators confirm those differences for the host species, but show a clear trend of higher diversity at the Makiling site compared with the trees from Palay–Palay site. This possible site effect could be due to the differing climate (higher temperature and humidity at Mt. Makiling; Salibay and Luyon 2008; Balete 2010; Combalicer and Im 2012). Interestingly, the low number of xylariaceous species (Table 1) isolated in the Philippines and contrastingly their high recovery from a Japanese subtropical forest (Ikeda et al. 2014) supports the assumption that tropical xylariaceous endophytes are less frequent in warmer tropical environments. However, the influence of abiotic environmental parameters must remain widely speculative at this point.

4.2. *Phylogeny and taxonomy*

The large proportion of Ascomycota and comparatively few Basidiomycota generally agrees with results from previously studied tropical endophytes (Arnold and Lutzoni 2007; Gazis and Chaverri 2010; Vaz et al. 2014b). Interestingly, Martin et al. (2015) reported diverse basidiomycetous endophytes in the populations of wild and planted rubber tree *Hevea*. On the other hand, the phylogenetic analysis revealed interesting patterns in the clustering of OTUs. First, more than a third of all observed OTUs were assigned to the Mycosphaerellaceae, in particular to *Pseudocercospora* and some closely related anamorphic genera. To the extent of our knowledge, such dominance of a single taxa was rarely observed during cultivation-based studies of leaf-inhabiting endophytes and could indicate special adaptations to establish themselves as endophytes of *Ficus* hosts.

Second, a relative yeast-rich community (6 genera, 6 OTUs) was identified. Ascomycete yeasts from the Saccharomycetales and several OTUs from the basidiomycete orders Cystofilobasidiales and Ustilaginales confirmed earlier trends (Solis et al. 2015). In their study on endophytes of the same *Ficus* host species from German greenhouses, they isolated a remarkable assemblage of pink and white basidiomycete

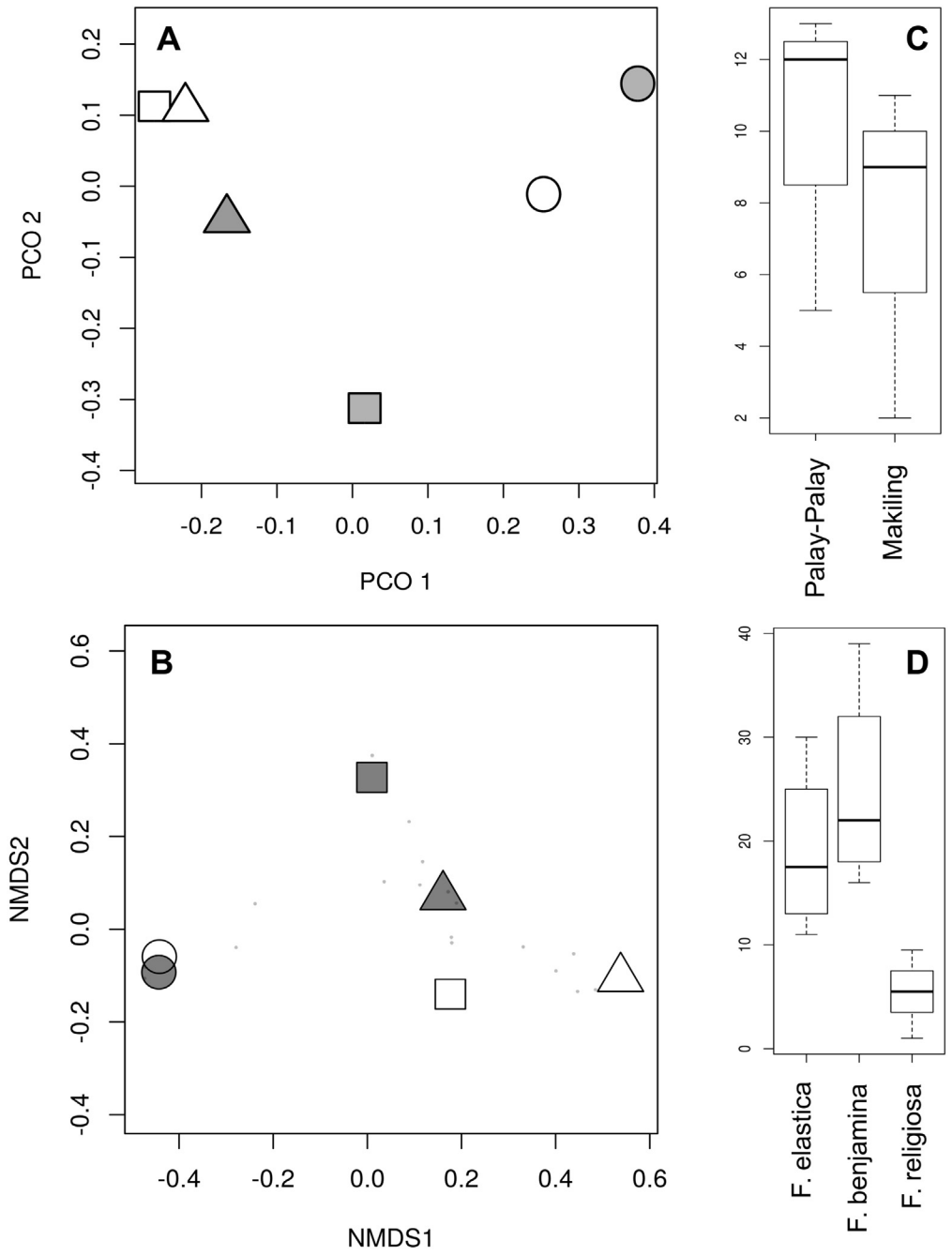

Fig. 4 – Fungal community composition is displayed for principal coordinates analysis (PCO, A) and non-metric multidimensional scaling (NMDS, B). In both ordinations, black symbols indicate origin from the site "Makiling", white symbols from "Palay–Palay". Circles indicate samples from *Ficus religiosa*, triangles from *F. benjamina* and squares from *F. elastica*. Box–Whisker plots in C show analysis of similarities (anosim) for the factor "site", D displays results for the factor "host".

yeasts, which was interpreted as a particular adaptation to the latex fluids of the plant tissues.

Latex is described as a vascular liquid enriched with various substances in solution or suspensions, such as sugars, starch grains, organic acids, sterols, protein, enzymes, amino acids, vitamins, lipids (Shukla and Krishna Murti 1971; Pallardy 2008; Lansky and Paavilainen 2011). While many studies explored pharmacologically important chemical compounds of leaf latex in many *Ficus* species (Burkill 1985; Nagaraju and Rao 1990; Holdsworth and Balun 1992), available data comparing the chemical profile of the three *Ficus* hosts is still lacking. Presently, the ecological role of latex is

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unknown therefore any assertion of influence to fungal endophyte communities remains speculative at this time. However, the presence of active compounds and defensive enzymes (Taira et al. 2005; Konno 2011) is of potential interest as this could potentially facilitate the selection of fungal inoculum.

4.3. *Community composition*

In contrast to the results from diversity analysis, a clear separation of the fungal assemblages was not detected between the two mountain sites. This observation however does not necessarily reflect the cosmopolitan nature of *Ficus* endophytes in Philippine forests especially with the lack of data describing the fungal endophyte communities of the surrounding vegetation. The non-uniform but rather patchy distribution of *Ficus* host trees within the forest collection sites suggest that these sparse hosts are colonized by generalists rather than specialists (May 1991). Our data showed the recovery of both few frequently-occurring and several rarely occurring fungi which implies that strict-sense generalism is not consistently the rule in environments with high plant diversity (Murali et al. 2007). Although dominance of endophyte species has been attributed to ecological factors (i.e., forest type, rainfall) (Suryanarayanan et al. 2011), the dominant taxa in this study (e.g., *Diaporthe*, *Phyllosticta*) are commonly encountered on many hosts of different tropical regions (e.g., India, Thailand, Brazil) (reviewed by Suryanarayanan et al. 2011). Contrastingly, the most prevalent taxa *Pseudocercospora* spp. isolated from the *Ficus* species targeted/sampled in this study have not been reported as prevalent fungal endophytes (de Abreu et al. 2010; Vaz et al. 2014b).

4.4. *Influence of host-identity on fungal endophyte communities*

Multi-gene phylogeny of *Ficus* species grouped *F. benjamina* and *F. elastica* as neighbouring taxa into section *Conosycea* and *F. religiosa* separately into section *Urostigma* (Rønsted et al. 2008). Notably, we observed a decreasing similarity of fungal communities with increasing phylogenetic distance of the host species, although no particular indicator taxa were detected for the individual hosts. Our results support the hypothesis that the evolutionary history of host plants is an important driver of fungal community composition including foliar endophytes of tropical plants (Whitman et al. 2012; Kembel and Mueller 2014). Our results stand in line with earlier observations that the similarity of fungal endophyte assemblage increased with phylogenetic proximity among hosts (Gilbert et al. 2007; Sieber 2007; Hoffman and Arnold 2008; Webb et al. 2008), whereas this correlation also extends into higher levels of host taxonomy as well (U'Ren et al. 2012; Kembel and Mueller 2014).

Our results being preliminary and limited in nature adequately supports the influence of host-related factors (e.g., phylogeny, taxonomy, traits) on observed fungal diversity, although more data are necessary to make such assertions conclusive.

Disclosure

The authors declare no conflict of interest. All the research activities undertaken in this study comply with the current laws of the Philippines and Germany.

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Appendix A. Supplementary material

Supplementary material related to this article can be found at http://dx.doi.org/10.1016/j.myc.2015.10.002.

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Biodiversity and Conservation

Cultivated leaf-inhabiting endophytic fungi from fig tree species (Ficus spp.) in German tropical greenhouses form distinct communities compared with natural outdoor conditions of the Philippines

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Corresponding Author:	Martin Unterseher, Ph.D Ernst-Moritz-Arndt University Greifswald Greifswald, GERMANY
Corresponding Author Secondary Information:	
Corresponding Author's Institution:	Ernst-Moritz-Arndt University Greifswald
Corresponding Author's Secondary Institution:	
First Author:	Michael Jay Lao Solis
First Author Secondary Information:	
Order of Authors:	Michael Jay Lao Solis
	Thomas Edison dela Cruz
	Martin Unterseher, Ph.D
Order of Authors Secondary Information:	
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Abstract:	Our understanding of leaf-associated fungal endophyte biodiversity from various host species has increased steadily over the years. Whereas artificial environments such as tropical greenhouses in temperate climate zones have become essential botanical repositories worldwide, our understanding of mycobiome diversity and composition under such conditions is poor and comparative studies with naturally occurring plants are almost not existent. In the present, cultivation-based study we assessed endophyte biodiversity in leaves of three Ficus species (F. religiosa, F. benjamina, F. elastica) from German tropical greenhouses (botanical gardens in Berlin and Greifswald) and from natural outdoor environments in the Philippines (Luzon island). Data analysis of the internal transcribed spacer rDNA region revealed significantly distinct fungal communities between natural and greenhouse samples, as well as non-overlapping species composition between the two greenhouse sites. The present results could add important understanding of the dynamics of plant-pathogens and invasive microorganisms in worldwide plant transportation.
Suggested Reviewers:	Derek Persoh University of Bochum derek.persoh@rub.de
	Samantha Karunarathna Mae Fah Luang University samanthakarunarathna@gmail.com
	Ondřej Koukol Charles University in Prague

	ondrej.koukol@natur.cuni.cz
	Jose Macia-Vicente Goethe-Universitat Frankfurt am Main MaciaVicente@em.uni-frankfurt.de

Cultivated leaf-inhabiting endophytic fungi from fig tree species (*Ficus* spp.) in German tropical greenhouses form distinct communities compared with natural outdoor conditions of the Philippines

Michael Jay. L. Solis[1,2], Thomas Edison dela Cruz[3] & Martin Unterseher[1*]

Affiliations:

[1] Ernst-Moritz-Arndt University Greifswald, Institute of Botany and Landscape Ecology, Soldmannstr. 15, 17487 Greifswald, Germany

[2] Department of Natural Sciences, College of Science and Information Technology, Ateneo de Zamboanga University, La Purisima St. 7000 Zamboanga City, Philippines

[3] Department of Biological Sciences, College of Science, University of Santo Tomas, España 1015 Manila, Philippines

*corresponding author: martin.unterseher@uni-greifswald.de, tel. +49 (0)3834 / 864184, fax. +49 (0)3834 / 864114

Abstract

Our understanding of leaf-associated fungal endophyte biodiversity from various host species has increased steadily over the years. Whereas artificial environments such as tropical greenhouses in temperate climate zones have become essential botanical repositories worldwide, our understanding of mycobiome diversity and composition under such conditions is poor and comparative studies with naturally occurring plants are almost not existent. In the present, cultivation-based study we assessed endophyte biodiversity in leaves of three *Ficus* species (*F. religiosa, F. benjamina, F. elastica*) from German tropical greenhouses (botanical gardens in Berlin and Greifswald) and from natural outdoor environments in the Philippines (Luzon island). Data analysis of the internal transcribed spacer rDNA region revealed significantly distinct fungal communities between natural and greenhouse samples, as well as non-overlapping species composition between the two greenhouse sites. The present results could add important understanding of the dynamics of plant-pathogens and invasive microorganisms in worldwide plant transportation.

Keywords: *Pseudocercospora* sp.; tropical greenhouses; plant pathogens; invasive species; fungal endophytes

Introduction

It is widely known that biodiversity of fungal endophytes differ among different host plants and biogeographic regions (U'Ren et al. 2012). However, several studies also reported a large proportion of shared taxa from different host plants and a generally large amount of ubiquistic species among endophytically occurring fungi (Unterseher et al. 2013a; Langenfeld et al. 2013; Blaadid et al. 2014; Solis et al. 2014). In these studies similarity of endophyte communities was partly attributed to similar climatic and environmental conditions. Conversely it was shown that endophytic communities from conspecific or congeneric host plants were different when analysed in different habitats (Leite et al. 2013; Unterseher et al. 2013b; Tateno et al. 2015; Martin et al. 2015). Consequently, these authors suggested strong structuring effects of geographic distance and climatic conditions on endophytic mycobiomes (Scholtysik et al. 2012; Higgins et al. 2014; Ikeda et al. 2014). Environmental conditions such as precipitation (Lau et al. 2013), temperature (Hashizume et al. 2010; Semmartin et al. 2015), elevation (Coince et al. 2014) and seasonality (Terhonen et al. 2011; Mishra et al. 2012) are among widely known drivers of diversity and community composition of fungal endophytes.

In addition, it is the ecology of fungal endophytes and their host plants that strongly impacts the observed community. Some plants (ie. grasses) are predominantly and systemically colonized by closely-related fungi (ie. clavicipitous fungi) (Murphy et al. 2015; Hodgson et al. 2015), whereas other hosts are mainly colonised from the surrounding vegetation and organic substrate (Miller et al. 2009; Vazquez de Aldana et al. 2013).

Greenhouses are artificial habitats providing uniform (i.e. mostly wind still, diffuse light conditions, identical temperature) and controlled conditions for all plants under the same roof (Jewett and Jarvis 2001; Zhang 2003). Traditionally, greenhouses are utilised for research in crop technology (Buttaro et al. 2015; Sanchez-Guerrero et al. 2015, Suzuki et al. 2015;

Messinger et al. 2015), effects of insecticides (Chen et al. 2015; Derksen et al. 2015), pest management (Cocco et al. 2015; da Camara et al. 2015), food pathogens (Diao et al. 2015), biotechnology (Frootan et al. 2015; Gimpel et al. 2015), seedling performance (Ha Duy et al. 2015; Lu et al. 2015) or microbial control (Holvoet et al. 2015; Oliver et al. 2015). Ecologically, greenhouses are recognised as important habitats (Davies et al. 2004, Lopez-Vaamonde et al. 2010). Despite the great ecological and economic interest of plant performance in such artificial ecosystems, studies about the significance of associated microorganisms and microfauna are still scarce (Paquin et al. 2008). Kenis (2009) and Kenis and Branco (2010) reported that arthropods and other plant pests in greenhouses originating from the tropics and subtropics affect biodiversity through various mechanisms, such as herbivory, predation, parasitism, competition and as disease vectors. Fungus-plant interactions in tropical greenhouses may deviate from those under natural or outdoor conditions, especially with respect to the non-natural distribution of vegetation within these closed habitats. For instance, sources of fungal inoculum in European greenhouses are often introduced as host plant contaminants (Hulme et al. 2008; Desprez-Loustau 2009). The introduction of non-native fungal pathogens has serious implications on the present quarantine protocols for transporting living species in Europe (Desprez-Loustau 2009). This study was conducted to assess and compare diversity and community composition of leaf-inhabiting endophytes of three *Ficus* tree species from tropical greenhouses in Germany and natural outdoor habitats in the Philippines (Solis et al. 2014, 2016). Specifically, we hypothesise a prominent effect of the locality (greenhouse vs. outdoor) on endophyte diversity and composition. Additionally we seeked to shed light on fungal host preferences in dependence of the environmental conditions of their host plants.

Materials and methods

Host species

With approximately 750 distinguished species, the monoecious genus *Ficus* belong to a large genus of vascular plants with tropical-subtropical distribution. The three investigated *Ficus* hosts *F. benjamina*, *F. elastica* and *F. religiosa* predominantly occur in tropical Asia-Pacific regions (eg. Nepal, Bhutan, India, Indonesia, China, Philippines, or tropical Australia). Furthermore, these species are widely cultivated ornamental plants in tropical greenhouses. *Ficus benjamina* is one of the most common office and indoor plants outside the tropics although it is known to cause hypersensitivity reactions of people exposed to latex and other allergens of this plant (Delbourg et al. 1995). *Ficus* leaves are distinctly evergreen; however habit and morphology (e.g. thickness and leaf dimensions) differ among species. *Ficus elastica* (thick, oblong leaves, 35 × 17 cm) and *F. religiosa* (broadly ovate to ovateorbiculate leaves, 7 × 4 cm) typically grow up to 30 m height while *F. benjamina* (oblong, elliptic, lanceolate, or ovate leaves, 4 × 1.5 cm) typically reaches heights of approx. 10 m.

Study sites

The collection sites in Germany are located in the tropical greenhouses of the botanical gardens of Berlin and Greifswald (Solis et al. 2014). Both sites are 250 km apart and are considered as two independent replicates (Figure 1). Outdoor collection sites included Mt. Makiling (eastern slope), Los Baños, Laguna; Mt. Palay-Palay/Mataas na Gulod National Park, Ternate, Cavite; and Manila City. Mount Makiling (4224 ha, elevation 1109 m; 65 km south of Manila City) and Mt. Palay-Palay/Mataas na Gulod National Park (4000 ha, elevation 648 m; 60 km southeast of Manila and 60 km northwest of Mt. Makiling) is comprised of primary and secondary forests (Solis et al. 2016). Manila City (3354 ha elevation 16 m) is a highly urbanised and industrialised area with patches of urban forests and

gardens. Given the distance of around 40 km among the three sites, they too are considered independent replicates within this investigation area (Figure 1).

Field work and sample processing

Collection and processing of the leaves followed identical protocols for both greenhouse and outdoor samples and are published elsewhere in detail (Solis et al. 2014, 2016). Sampling was done from November 2012 to March 2013. Samples from collection sites within short-distances to the laboratory were transported in sterile bags inside coolers and processed immediately, otherwise leaf samples were stored at 4 ºC and processed within 12 h. Leaves were then thoroughly rinsed with sterile distilled water to remove any dirt and debris and surface sterilised by submerging into 70 % ethanol for 2 min, sodium hypochloride (1 % active chloride) for 5 min, and 70 % ethanol for 1 min respectively. Four plugs per leaf with 10 mm diameter were produced with a sterile cork borer under sterile conditions. One hundred leaf plugs per tree species and site were further homogenized according to the dilution-to-extinction protocol for foliar endophytes (Unterseher et al. 2013b) and plated onto sterile 48-well multiwell plates (Carl Roth, Karlsruhe, Germany) containing 1.5 % Malt Extract Agar (MEA) supplemented with Tetracycline (10 mg l^{-1}) and Cyclosporine (10 µl l^{-1}). The plates were examined regularly for 30 days and emerging fungal colonies were axenically transferred onto fresh MEA plates for morphological analysis, morphotype grouping and DNA extraction. Duplicates of the Philippine cultures were shipped to Greifswald and strictly followed a memorandum of cooperation and a material transfer agreement signed by the two universities of Greifswald and Manila. Vouchers are deposited in both Universities and in parts in the German Collection of Microorganisms and Cell Cultures (DSMZ).

DNA extraction, amplification and sequencing

Fungal genomic DNA was extracted using common CTAB protocols and the commercially available Master Pure Yeast DNA Purification Kit (epicentre, Madison, U.S.A). Amplification of the internal transcribed spacer (ITS) region was performed with primers ITS1 and ITS4 (White et al. 1990) and chemicals from the MangoTaq DNA Polymerase kit (Bioline, London, UK) (15.64 µl ddH_20, 5 µl Mango-Buffer, 1.7 µl $MgCl_2$ (50 mM), 0.5 µl dNTP (10 mM), 0.5 µl of each primer (10 pM / µl), 0.16 µl (5 U / µl) of Taq DNA polymerase and 1 µl template DNA; (5 min at 94ºC, followed by 35 cycles (35 s at 94 ºC, 50 s at 52 ºC, 1 min 30 s at 72 ºC), and 5 min at 72 ºC. PCR products were run on agarose gel (0.8 %) for control. The unpurified amplicons were shipped to Beckman Coulter Genomics (Takeley, UK) for sequencing. Chromatograms were manually inspected to eliminate erroneous sequences. and remnants of the flanking 18S and 28S ribosomal RNA gene were removed with ITSx (Bengtsson-Palme et al. 2013). Sequences were deposited under accessions LN997656-LN997800. Taxon assignments and other basic data are provided below and as online supporting information.

OTU clustering

The ITS1 region was used for OTU clustering due to its higher sequences variability and higher efficiency for species discrimination compared with ITS2 (Wang et al. 2015). QIIME "pick_otus.py" function was used (Navas-Molina et al. 2013) with default settings at 90, 95, 97 and 98.5 % sequence similarity thresholds. Sequence processing continued with extraction of representative sequences, taxon annotation with the UNITE reference data base (Koljalg et al. 2013) and computing of final OTU tables. In order to decide about the most appropriate OTU threshold for taxonomic assignment of the filamentous fungi, we compared the different OTU groupings with morphotype information, ITS phylogeny and BLAST searches.

Phylogenetic analysis

ITS1 and 5.8S regions were extracted from the full-length representative ITS sequences and aligned with MAFFT 6 using the E-INS-i strategy (Katoh and Toh 2008) with slight manual refinement in Mesquite version 2.75 (Maddison and Maddison 2011). Phylogenetic reconstruction was conducted using Maximum Likelihood (ML) and Bayesian Interference (BI). ML was run on raxmlGUI (Silvestro and Michalak 2012) using the rapid bootstrap option with 1,000 replicates. For Bayesian analysis the appropriate model for minimum evolution was selected from the 24 models implemented in MrModeltest 2.1 (Nylander 2004). 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 5 Mio., 1,000 and 0.25 respectively. Clade support was assessed with posterior probabilities.

Assessment of richness and community composition

The QIIME-generated abundance-based OTU tables were used for analysis of species richness and community composition. Species richness analysis for OTUs used mathematically smoothed (ie. randomized) species accumulation curves to display the accumulation of "species" when the number of records increases (Gotelli and Colwell 2001). By analysing the shape of the curves (eg. initial slope, approaching an asymptote or not), it was possible to evaluate basic patterns of species richness (observed species richness, sampling depth) independently from differing sampling effort among samples or sample groups.

Community composition of fungal endophytes was assessed with nonmetric multidimensional scaling (NMDS) and principal coordinate analysis (PCO; PCoA) based on

the "Cao" dissimilarity metrics (Cao et al. 1997) which was described as a minimally biased index for high beta diversity and variable sampling intensity. The distinctiveness of endophyte assemblages from greenhouses and outdoor habitats and from the three host species *F. benjamina*, *F. elastica* and *F. religiosa* was tested with PERMANOVA (permutational multivariate analysis of variance using distance matrices).

Results

Host and site-based species richness

A total of 2,387 isolates were recovered from the 6 collection sites (see Table 1). An accurate morphotype grouping for similar-looking strains was created based on colony characters (ie. colony colour, colony margin, colony shape, spore size, spore shape, presence of growth rings, and growth speed). and resulted in 32 morphotypes from Germany and 297 from the Philippines. Morphotypes with 10 or less isolates were all included in ITS sequencing while a maximum of 10 isolates were selected for morphotypes with more than 10 isolates. In total, 788 representative strains were selected for ITS sequencing. Removal of sequences with poor chromatogram signals resulted in 654 full-length high-quality ITS sequences. OTU clustering of the ITS1 region at 90, 95, 97 and 98.5 % sequence similarity resulted in 90, 151, 243 and 377 OTUs, respectively. The 97 % similarity threshold represented the most appropriate compromise between morphotype grouping, BLAST analysis and phylogeny. The community data derived from this OTU clustering were used for subsequent assessment of endophytic biodiversity.

Exploratory data analysis revealed a strong positive correlation of OTU richness and number of sequences, thus the more sequences were obtained from a sample or sample group the

more OTUs were observed (not shown). Rarefaction was therefore applied for species richness analysis (Fig. 2). The species accumulation curves for the three host species display almost linear increase in richness. Curve shape of all three *Ficus* species was similar (Fig. 2A). Based on the present data, Chao 2 estimator calculated 205 ± 43 OTUs for *F. benjamina*, 186 ± 40 OTUs for *F. elastica* and 65 ± 25 OTUs for *F. religiosa*. The two rarefied species accumulation curves for all combined greenhouse and outdoor samples displayed a clear trend of lower richness of fungal endophytes in greenhouse plants than in plants growing in outdoor conditions (Fig. 2B). Corresponding Chao 2 estimators resulted in 106 ± 25 OTUs for the first and 253 ± 66 OTUs for the latter sample group.

Taxonomy and phylogeny of fungal endophytes

Figure 3 displays the results from maximum-likelihood analysis of the ITS1-5.8S gene region from representative sequences of the 151 OTUs. The tree displays well-supported monophyly (≥ 70 % bootstrap and post. prob. support) of main asco- and basidiomycete orders. Bayesian analysis confirmed this topology (not shown). The most diverse order was Capnodiales with 51 OTUs (Fig. 3). The two families Mycophaerellaceae and Davidiellaceae separated with high statistical support. Figure 3 displays additionally the presence of OTUs in the three host species and the two sample environments. Members of the Mycosphaerellaceae were almost exclusively isolated from the outdoor environments of the Philippines, whereas OTU richness and abundance of the Davidiellaceae (e.g. *Cladosporium*) were higher in samples from German greenhouses. Isolates belonging to the Helotiales, Hypocreales (both Ascomycota), Tremellales and Cystofilobasidiales (both Basidiomycota) were more frequent in greenhouse samples, Glomerellales, Diaporthales (both Ascomycota) and Polyporales (Basidiomycota) were more frequent in outdoor samples (Fig. 3).

Endophytic community composition

Both NMDS and PCO ordinations showed contrasting community composition between the botanical greenhouses in Germany and the natural habitats/outdoor environments in the Philippines (Fig. 4). The endophytic community was significantly related to the type of environment (PERMANOVA F = 3.54, R^2 = 0.22, p = 0.001) whereas the influence of host species in shaping the endophytic communities remained without sufficient statistical support (PERMANOVA F = 1.06, R^2 = 0.13, p = 0.326). The minor influence of host identity is clearly visible in the ordinations (Fig. 4). There the ellipsoids around the centroids of both *F. benjamina* (green solid line) and *F. elastica* (brown dashed line) samples are strongly overlapping. Endophytic composition of *F. religiosa* samples (blue diamonds in Fig. 4A and B) differed from the other two leading to a different position of its centroid (ellipsoid with blue dotted line).

All German samples showed low dispersion in ordination space, suggesting a more homogenous composition of its endophytic community, compared with the outdoor samples, which were broadly distributed in ordination space. When analysing sub data from Germany and the Philippines separately, the effect of sample origin became significant for the first (Greifswald vs. Berlin, PERMANOVA F = 3.1, R^2 = 0.4, p = 0.001) but not for the second data set (Mt. Makiling vs. Los Baños vs. Manila; PERMANOVA F = 1.17, R^2 = 0.26, p = 0.147).

Discussion

Richness of* Ficus *endophytes

Natural outdoor samples from the Philippines revealed higher OTU richness than the greenhouse samples, with the species accumulation curves of the latter bending towards the asymptote at clearly lower values (Fig. 2). Fungal endophytes in the tropics are

comparatively well-studied and known to form diverse communities with low host preferences (Suryanarayanan et al. 2011, Gazis & Chaverri 2015). In contrast, fungal endophytes of tropical plants in artificial environments such as climate-controlled greenhouses are almost unexplored so far. In a recent study about root endophytes, a strong decrease in fungal richness was observed when comparing the plant *Anemopsis californica* in the wild (11 OTUs) with plants grown for one year in a greenhouse (2 OTUs) (Bussey et al. 2015). Considering the predominant horizontal transmission of most fungal endophytes (Rodriguez et al. 2009), the lower fungal richness in plants from greenhouses observed in the present study is likely due to limited spore sources coming from a less diverse indoor vegetation. Additionally various control measures such as spraying of pesticides, watering with filtered water, mounting of insect traps might further lead to a decrease of fungal infection rates. In the present study the lowest observed OTU richness from trees in Manila megacity was clearly attributed to the low sampling intensity. However several studies have shown a likewise negative influence of urbanisation on the diversity of fungal endophytes (Kaye et al. 2006; Jumpponen and Jones 2010; Matsumura and Fukuda 2013) which is obviously due to a variety of environmental stressors, such as high concentration of aerial pollutants, habitat fragmentation, stand isolation or constant litter removal.

Community composition of* Ficus *endophytes

The combined analysis of phylogenetic composition of the endophyte assemblage and OTU occurrence (Fig. 3) revelaed that Mycosphaerellaceae lineage, in particular *Pseudocercospora* and some closely related anamorphic genera formed the largest clade and exclusively represented isolates from the tropical outdoor environments. The differing species composition between greenhouse and outdoor samples infered from Fig. 3 became even more obvious when analysing results of multivariate analysis (Fig. 4a), thus confirming the earlier

raised hypothesis. With respect to the observed tropical Mycosphaerellaceae clade in Fig. 3, available literature and the authors' knowledge suggest that the plant-pathogenic genus *Pseudocercospora* and further seven genera obtained exclusively from the Philippine samples (*Meyerozyma*, *Phyllosticta*, *Pseudozyma*, *Wrightoporia*, *Zasmidium*, *Stenella*, *Leptosphaerulina*) have not been reported so far from Europe (eg. Seifert et al. 2011). The apparent absence of these fungal genera in Europe could indicate that the temperate climate has a structuring influence preventing a stable establishment of these taxa in natural endophyte communities. Further endophyte cultures of *Ficus* had high sequence similarity with *Lentinus* and *Phaeosphaeria*, the first being a known tropical mushroom (Karunarathna et al. 2011) while the latter a plant pathogen reported as invasive species to Europe (Desprez-Loustau 2009) were both isolated from German and Philippine samples. The potential of botanical gardens as pathways for the entry of pathogenic and invasive ones co-transported with living plants (Brasier 2008) are of important ecological and economic concern. A clear bipartion of yeast-like endophytes was observed. Basidiomycete yeasts with affiliations to Tremellales or Filobasidiales (mainly *Rhodotorula*, *Cryptococcus*) were not isolated from the tropical outdoor samples. Contrarily, members of the Saccharomycetales (the "asco-yeasts") were not observed in indoor cultures.

Transformation of cultures into molecular data

Cultivation approaches for the analysis of fungal communities such as the present study typically contain several independent sampling and isolation events followed by a grouping of the cultures according to their growth characteristics in Petri dishes on artificial media (Lacap et al. 2003, Unterseher et al. 2013a,b, Gazis & Chaverri 2015, Solis et al. 2016). Generally speaking, the higher the number of axenic cultures the stronger the need for selecting a representative set for conventional sequencing (this study, but see Gazis &

Chaverri 2015 for a complete molecular representation of cultivated strains). In few cases only, the original abundance of the entire culture data set can be restored, if morphotyping and OTU clustering equates each other (Unterseher & Schnittler 2010). However cultures of fungal endophytes are often hard to tell apart according to their growth characteristics (e.g. non sporulating, whitish and cottony). Numerous options for OTU clustering and the choice of sequence similarity thresholds further complicates or even prohibits the assignment of non-sequenced cultures to established OTUs. In the present study, the original information of culture data was strongly reduced as a consequence of tight personal and financial constraints. Given that morphotype richness, but not abundance distribution was preserved in the molecular data, this paper should be viewed as case study with only few results allowing for generalisations. Bearing in mind such general limitations of cultivation studies, priority should be given to cultivation-independent multiplexed high throughput sequencing (Persoh 2015, Unterseher et al. 2016).

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Tables and figures

Table 1. List of OTUs obtained after clustering at 97% similarity and taxonomic assignment using BLAST and UNITE; Fr - *Ficus religiosa*, Fe - *F. elastica*, Fb - *F. benjamina*.

Figure 1. *Ficus* hosts collection sites in Germany (Greifswald and Berlin) and in the Philippines (Manila, Mt. Palay-Palay, Mt. Makiling).

Figure 2: Species accumulation curves of fungal endophytes from the three host plants (A) and from the two origins of leaf samples (B).

Figure 3: Phylogenetic analysis of *Ficus* endophytes is combined with OTU occurrence. Topology of the tree is based on Maximum-Likelihood (ML) calculations. Thickened branches indicate support above 0.7 for both ML and Bayesian analysis. The "Mycosphaerellaceae-gap" for greenhouse samples is most obvious. Members of the Helotiales and Tremellales were absent from the tropical endophyte assemblage.

Figure 4: Community composition of *Ficus* endophytes based on NMDS (A) and PCO (B). Samples from outdoor and greenhouse locations are connected with each other and labelled accordingly at their centroids. Samples from the three host species are displayed in different symbols and colours (*F.* benjamina: green, dots, solid ellipsoids; *F.* elastica: brown, squares, dashed ellipsoids; *F. religiosa*: blue, rhombs, dotted ellipsoids). The dispersion of host specific samples is displayed as ellipsoids around the corresponding centroids with a standard deviation of 0.95.

Electronical supplementary material

ESM01: This folder contains the ITS alignment and two tree files.

ESM02: This folder contains all input files and commands to perform the biodiversity

OTU	Taxon	Country	Site	Host	Abundance
3	Cadophora malorum	Germany	Berlin	Fr, Fe	6
4	Mycosphaerella	Germany	Berlin	Fe, Fb	38
75	Alternaria	Germany	Berlin	Fr, Fe, Fb	12
78	Penicillium	Germany	Berlin	Fr	2
79	Ascomycota	Germany	Berlin	Fr	2
83	Arthrinium	Germany	Berlin	Fr, Fb	14
106	Hypocreales	Germany	Berlin	Fb	2
107	Pezizomycotina	Germany	Berlin	Fb	4
109	Hypocreales	Germany	Berlin	Fb	2
111	Sordariomycetes	Germany	Berlin	Fe, Fb	8
112	Pezizomycotina	Germany	Berlin	Fe, Fb	8
113	Pezizomycotina	Germany	Berlin	Fb	2
114	Cryptococcus	Germany	Berlin	Fb	2
115	Penicillium	Germany	Berlin	Fb	2
116	Penicillium	Germany	Berlin	Fb	2
117	Rhodotorula	Germany	Berlin	Fb	2
130	Penicillium	Germany	Berlin	Fe	2
131	Hypocreales	Germany	Berlin	Fe	2
132	Hypocreales	Germany	Berlin	Fe	2
133	Hypocreales	Germany	Berlin	Fe	4
134	Alternaria	Germany	Berlin	Fe, FB	10
135	Cladosporium	Germany	Berlin	Fr, Fe	4
136	Phaeosphaeria	Germany	Berlin	Fr	2
137	Pezizomycotina	Germany	Berlin	Fr	2
138	Pezizomycotina	Germany	Berlin	Fe	2
139	Hypocreales	Germany	Berlin	Fe	2
143	Coniochaetaceae	Germany	Berlin	Fe	4
19	Cryptococcus	Germany	Greifswald	Fr, Fe, FB	18
46	Pezizomycotina	Germany	Greifswald	Fr, Fe, Fb	2
47	Cryptococcus	Germany	Greifswald	Fr	6
61	Dothideomycetes	Germany	Greifswald	Fb	2
63	Antrodia sinuosa	Germany	Greifswald	Fb	2
64	Rhodotorula	Germany	Greifswald	Fb	2
65	Hypocreales	Germany	Greifswald	Fr, Fe	54
66	Exophiala	Germany	Greifswald	Fb	6
68	Penicillium	Germany	Greifswald	Fb	2
69	Rhodotorula	Germany	Greifswald	Fb	2
74	Cryptococcus	Germany	Greifswald	Fb	2
82	Hypocreales	Germany	Greifswald	Fb	2
84	Rhodotorula	Germany	Greifswald	Fb	2
97	Rhodotorula	Germany	Greifswald	Fe	2
98	Cryptococcus	Germany	Greifswald	Fe	2
99	Bjerkandera	Germany	Greifswald	Fb	2

0	Rhodotorula mucilaginosa	Germany	Greifswald, Berlin	Fr, Fe, Fb	26
1	Aureobasidium sp.	Germany	Greifswald, Berlin	Fr, Fe, Fb	66
2	Rhodotorula	Germany	Greifswald, Berlin	Fr, Fe, Fb	50
24	Penicillium sp.	Germany	Greifswald, Berlin	Fr, Fe, Fb	22
57	Cryptococcus	Germany	Greifswald, Berlin	Fr, Fe, Fb	50
60	Exophiala	Germany	Greifswald, Berlin	Fb	8
62	Hypocreales sp.	Germany	Greifswald, Berlin	Fe, Fb	8
70	Rhodotorula	Germany	Greifswald, Berlin	Fe, Fb	4
80	Cladosporium	Germany	Greifswald, Berlin	Fr, Fe, Fb	20
28	Rhodotorula	Germany	Greifswald, Berlin	Fb	8
5	Penicillium	Philippines	Manila	Fe	4
17	Wrightoporia tropicalis	Philippines	Manila	Fr, Fe	12
110	Polyporaceae	Philippines	Manila	Fr	2
118	Mycosphaerella	Philippines	Manila	Fb	2
119	Pseudocercospora	Philippines	Manila	Fb	2
120	Phomopsis	Philippines	Manila	Fb	2
121	Bjerkandera adjusta	Philippines	Manila	Fb	2
122	Polyporales	Philippines	Manila	Fb	2
123	Aspergillus	Philippines	Manila	Fb	2
125	Hymenochaetaceae	Philippines	Manila	Fe	2
127	Polyporales	Philippines	Manila	Fe	2
129	Strophariaceae	Philippines	Manila	Fe	2
73	Mycosphaerellaceae sp.	Philippines	Mt. Makiling	Fe	4
9	Debaryomyces hansenii	Philippines	Mt. Makiling	Fe	4
11	Guehomyces pullulans	Philippines	Mt. Makiling	Fe	4
13	Aspergillus	Philippines	Mt. Makiling	Fe	4
15	Penicillium	Philippines	Mt. Makiling	Fb	4
20	Mycosphaerella	Philippines	Mt. Makiling	Fb	4
21	Mycosphaerella	Philippines	Mt. Makiling	Fe, Fb	24
22	Exophiala sp.	Philippines	Mt. Makiling	Fb	2
26	Lecythophora	Philippines	Mt. Makiling	Fe	8
88	Epicoccum	Philippines	Mt. Makiling	Fb	2
89	Leptosphaerulina	Philippines	Mt. Makiling	Fr	8
90	Periconia	Philippines	Mt. Makiling	Fr	2

Strain mo.	Accession no.	OTU	Taxon	Country
1130	LN997659	91	Diatrypaceae	Philippines
1329	LN997660	92	Diatrypaceae	Philippines
961	LN997720	93	Sporobolomyces	Philippines
935	LN997722	148	Pseudocercospora	Philippines
939	LN997723	149	Epicoccum	Philippines
926	LN997726	150	Pseudocercospora	Philippines
1258	LN997742	151	Exophiala	Philippines
1257	LN997743	152	Phaeosphaeria	Philippines
1245	LN997744	153	Pseudocercospora	Philippines
1280	LN997746	158	Pseudocercospora	Philippines
1197	LN997747	159	Cladosporium	Philippines
1193	LN997748	160	Phomopsis	Philippines
1198	LN997749	161	Cladosporium	Philippines
1237	LN997750	162	Eutypella	Philippines
1224a	LN997751	163	Epicoccum	Philippines
1224	LN997752	164	Pseudocercospora	Philippines
1090	LN997762	165	Basidioradulum	Philippines
1086	LN997763	166	Stenella musae	Philippines
1058	LN997764	167	Pseudocercospora	Philippines
1119	LN997765	168	Phyllosticta	Philippines
1117	LN997766	169	Mycosphaerella	Philippines
1031	LN997767	170	Mycosphaerella	Philippines
1035	LN997768	171	Colletotrichum	Philippines
1022	LN997769	172	Pseudocercospora	Philippines
1050	LN997770	173	Glomerella cingulata	Philippines
1047	LN997771	174	Pleosporales	Philippines
1136	LN997772	175	Pseudocercospora	Philippines
124	LN997674	176	Mycosphaerellaceae	Philippines
195	LN997698	178	Phomopsis sp.	Philippines
201	LN997699	179	Phomopsis sp.	Philippines
785	LN997709	16	Mycosphaerellaceae	Philippines
844	LN997711	27	Cryptococcus Filobasidium	Philippines
826	LN997712	30	Cystofilobasidiaceae	Philippines
81	LN997713	32	Penicillium	Philippines
821	LN997714	33	Aspergillus	Philippines
568	LN997715	34	Stenella musae	Philippines
589	LN997716	36	Aspergillus	Philippines
734	LN997719	37	Pseudocercospora	Philippines
853	LN997725	38	Pseudocercospora	Philippines
920	LN997727	40	Zasmidium scaevolicola	Philippines
326	LN997737	41	Mycosphaerella etlingerae	Philippines
509	LN997738	42	Pseudocercospora	Philippines
544	LN997739	43	Mycosphaerella sp.	Philippines

101	LN997656	48	Mycosphaerella	Philippines
1132	LN997657	49	Lentinus	Philippines
1128	LN997658	50	Mycosphaerella sp.	Philippines
1053	LN997679	54	Cladosporium	Philippines
211	LN997707	55	Pseudocercospora	Philippines
801	LN997708	85	Mycosphaerellaceae sp.	Philippines
758	LN997710	86	Mycosphaerellaceae sp.	Philippines
586	LN997717	87	Mycosphaerellaceae sp.	Philippines
932	LN997724	94	Pseudocercospora	Philippines
1820	LN997683	95	Phyllosticta	Philippines
1356	LN997661	96	Pseudocercospora	Philippines
1322a	LN997673	101	Pseudozyma Ustilago	Philippines
1315	LN997745	105	Pseudozyma Ustilago	Philippines
1403	LN997753	154	Meyerozyma guilliermondii	Philippines
1397a	LN997754	155	Aspergillus	Philippines
1420	LN997755	156	Phomopsis	Philippines
1419	LN997756	157	Phomopsis	Philippines
1415	LN997757	10	Mycosphaerellaceae sp.	Philippines
1408	LN997758	29	Pseudocercospora	Philippines
1346	LN997759	31	Glomerella cingulata	Philippines
1340	LN997760	35	Pseudocercospora	Philippines
1349	LN997761	52	Phyllosticta	Philippines
728	LN997718	147	Pseudocercospora	Philippines
1647	LN997665	23	Phyllosticta	Philippines
1702	LN997667	39	Pseudocercospora	Philippines
1693	LN997669	7	Mycosphaerella sp.	Philippines
2433	LN997671	8	Pseudocercospora	Philippines
1567	LN997675	12	Mycosphaerella	Philippines
1568	LN997676	25	Meyerozyma guilliermondii	Philippines
1604	LN997677	51	Pseudocercospora	Philippines
1720	LN997681	6	Epicoccum nigrum	Germany, Philippines
2350	LN997729	14	Cladosporium	Germany, Philippines
2502	LN997730	76	Cladosporium	Germany, Philippines
2501	LN997731			

Site	Host	Abundance	Strain mo.	Accession no.
Mt. Makiling	Fb	2	2266	LN997732
Mt. Makiling	Fb	2	2266	LN997732
Mt. Makiling	Fb	2	2267	LN997733
Mt. Makiling	Fe	2	1633	LN997773
Mt. Makiling	Fe	2	1691	LN997774
Mt. Makiling	Fe	2	1687	LN997775
Mt. Makiling	Fb	2	1600	LN997776
Mt. Makiling	Fb	2	1616	LN997777
Mt. Makiling	Fb	6	1607	LN997778
Mt. Makiling	Fe, Fb	6	1709	LN997782
Mt. Makiling	Fe	2	1708	LN997783
Mt. Makiling	Fe	2	1712	LN997784
Mt. Makiling	Fe	2	1708	LN997783
Mt. Makiling	Fe	2	1698	LN997785
Mt. Makiling	Fe	2	1707	LN997786
Mt. Makiling	Fe	2	1723	LN997787
Mt. Makiling	Fe	2	1726	LN997788
Mt. Makiling	Fb	2	1528	LN997789
Mt. Makiling	Fb	2	1516	LN997790
Mt. Makiling	Fb	2	1540	LN997791
Mt. Makiling	Fb	6	1497	LN997792
Mt. Makiling	Fb	2	1509c	LN997793
Mt. Makiling	Fb	2	1509b	LN997794
Mt. Makiling	Fb	2	1510	LN997795
Mt. Makiling	Fb	2	1506	LN997686
Mt. Makiling	Fb	4	1593	LN997796
Mt. Makiling	Fb	2	1582	LN997797
Mt. Makiling	Fb	6	1551	LN997798
Mt. Makiling	Fb	2	1558	LN997799
Mt. Makiling	Fb	2	1557	LN997800
Mt. Palay-Palay	Fe	8	2604	LN997672
Mt. Palay-Palay	Fe	4	2256	LN997682
Mt. Palay-Palay	Fe	4	2239	LN997685
Mt. Palay-Palay	Fb	4	1805	LN997687
Mt. Palay-Palay	Fe	2	2220	LN997688
Mt. Palay-Palay	Fe	4	2206	LN997689
Mt. Palay-Palay	Fe	4	2220	LN997688
Mt. Palay-Palay	Fe	2	2181	LN997691
Mt. Palay-Palay	Fe	12	2201	LN997692
Mt. Palay-Palay	Fe	2	2217	LN997694
Mt. Palay-Palay	Fe	2	2231	LN997695
Mt. Palay-Palay	Fe	2	2219	LN997696
Mt. Palay-Palay	Fe	2	2042	LN997697

Mt. Palay-Palay	Fe	4	2041	LN997700
Mt. Palay-Palay	Fe	4	2040	LN997701
Mt. Palay-Palay	Fe	2	2037	LN997702
Mt. Palay-Palay	Fe	2	2175	LN997705
Mt. Palay-Palay	Fe	2	2171	LN997706
Mt. Palay-Palay	Fe	2	2262	LN997728
Mt. Palay-Palay	Fe	2	2262	LN997728
Mt. Palay-Palay	Fe	2	2262	LN997728
Mt. Palay-Palay	Fe	2	2257	LN997734
Mt. Palay-Palay	Fe	2	2254	LN997735
Mt. Palay-Palay	Fe	2	2248	LN997736
Mt. Palay-Palay	Fr	2	2508	LN997740
Mt. Palay-Palay	Fe	2	2808	LN997741
Mt. Palay-Palay	Fb	10	1744	LN997779
Mt. Palay-Palay	Fb	2	1825	LN997780
Mt. Palay-Palay	Fb	2	1822	LN997781
Mt. Palay-Palay	Fb	2	1822	LN997781
Mt. Palay-Palay, Mt. Makiling	Fe, Fb	38	1671	LN997666
Mt. Palay-Palay, Mt. Makiling	Fe, FB	14	1819	LN997684
Mt. Palay-Palay, Mt. Makiling	Fe, Fb	16	1506	LN997686
Mt. Palay-Palay, Mt. Makiling	Fe, Fb	30	2223	LN997690
Mt. Palay-Palay, Mt. Makiling	Fe, Fb	12	2154	LN997704
Mt. Palay-Palay, Mt. Makiling	Fe, Fb	53	1633	LN997773
Mt. Palay-Palay, Mt. Makiling, Manila	Fr, Fe, Fb	24	2505	LN997678
Mt. Palay-Palay, Mt. Makiling, Manila	Fe, Fb	48	2215	LN997693
Mt. Palay-Palay, Mt. Makiling, Manila	Fe, FB	75	2120	LN997663
Mt. Palay-Palay, Mt. Makiling, Manila	Fe, FB	81	1563	LN997664
Mt. Palay-Palay, Mt. Makiling, Manila	Fe, FB	10	1550	LN997668
Mt. Palay-Palay, Mt. Makiling, Manila	Fe, Fb	12	1737	LN997680
Mt. Palay-Palay, Mt. Makiling, Manila	Fe, Fb	51	2099	LN997703
Berlin, Mt. Makiling, Manila	Fe, Fb	12	1357	LN997662
Berlin, Mt. Palay-Palay, Mt. Makiling, Manila	Fr, Fe, FB	14	2500	LN997670
Greifswald, Belin, Manila	Fr, Fe, Fb	18	960	LN997721

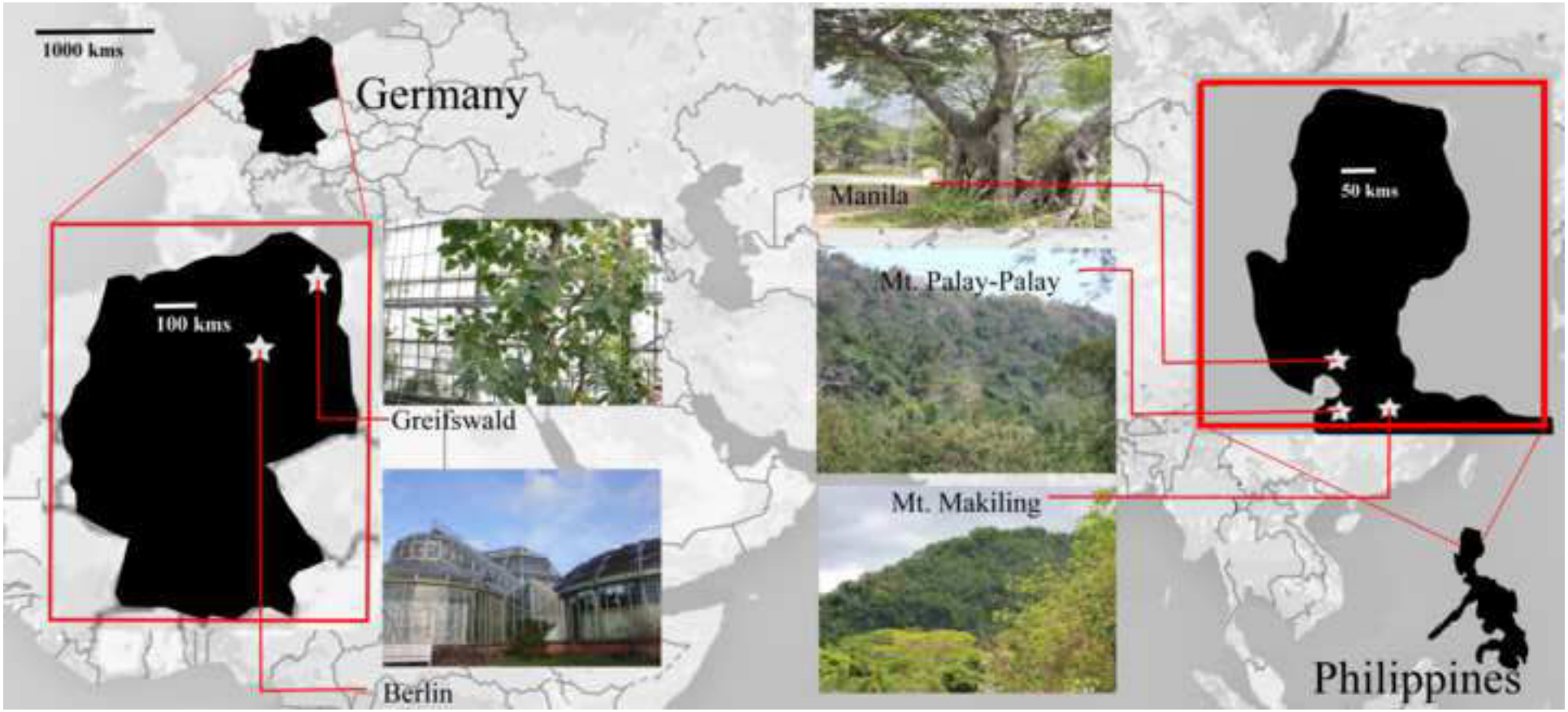

Figure 2

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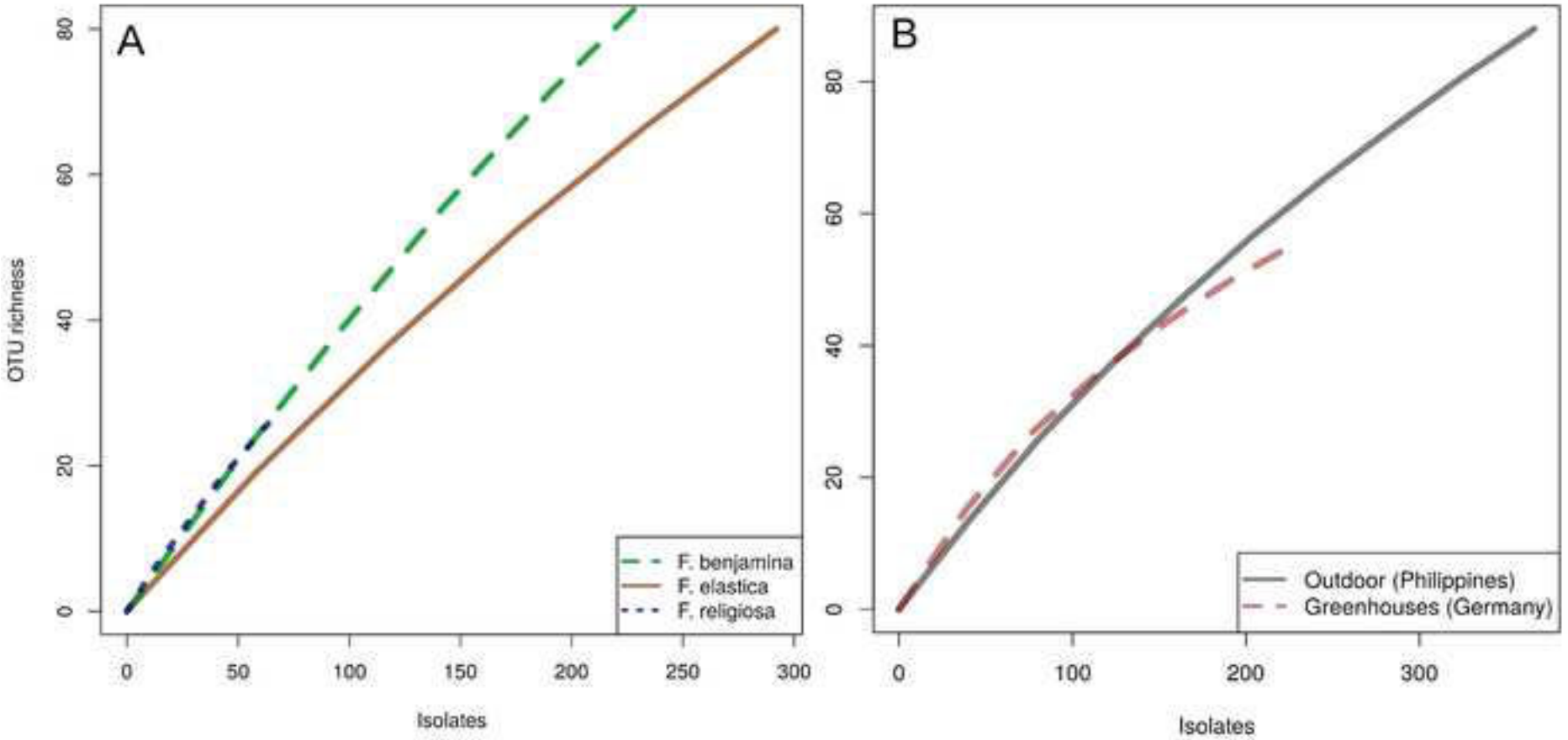

Figure 3

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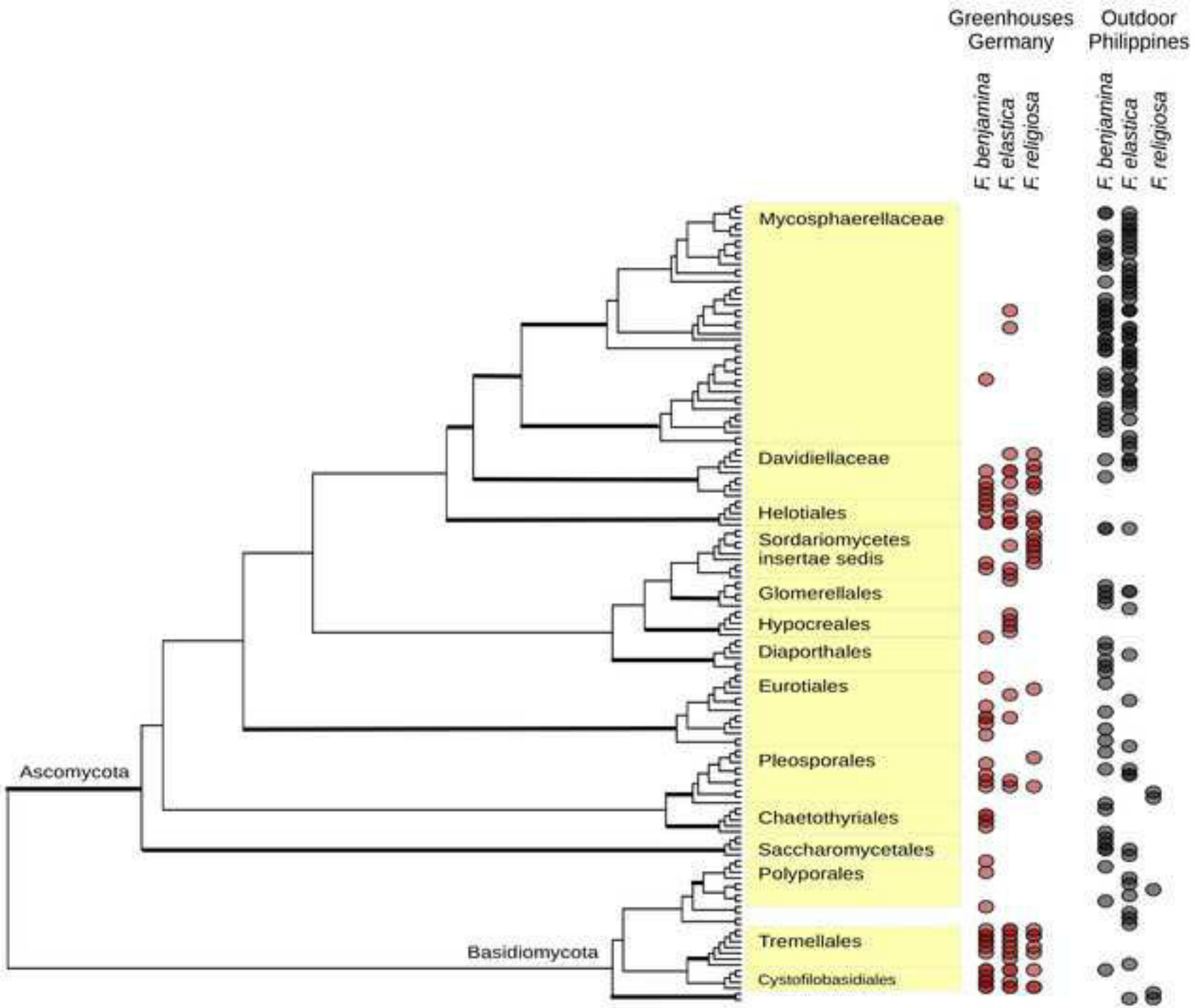

Figure 4

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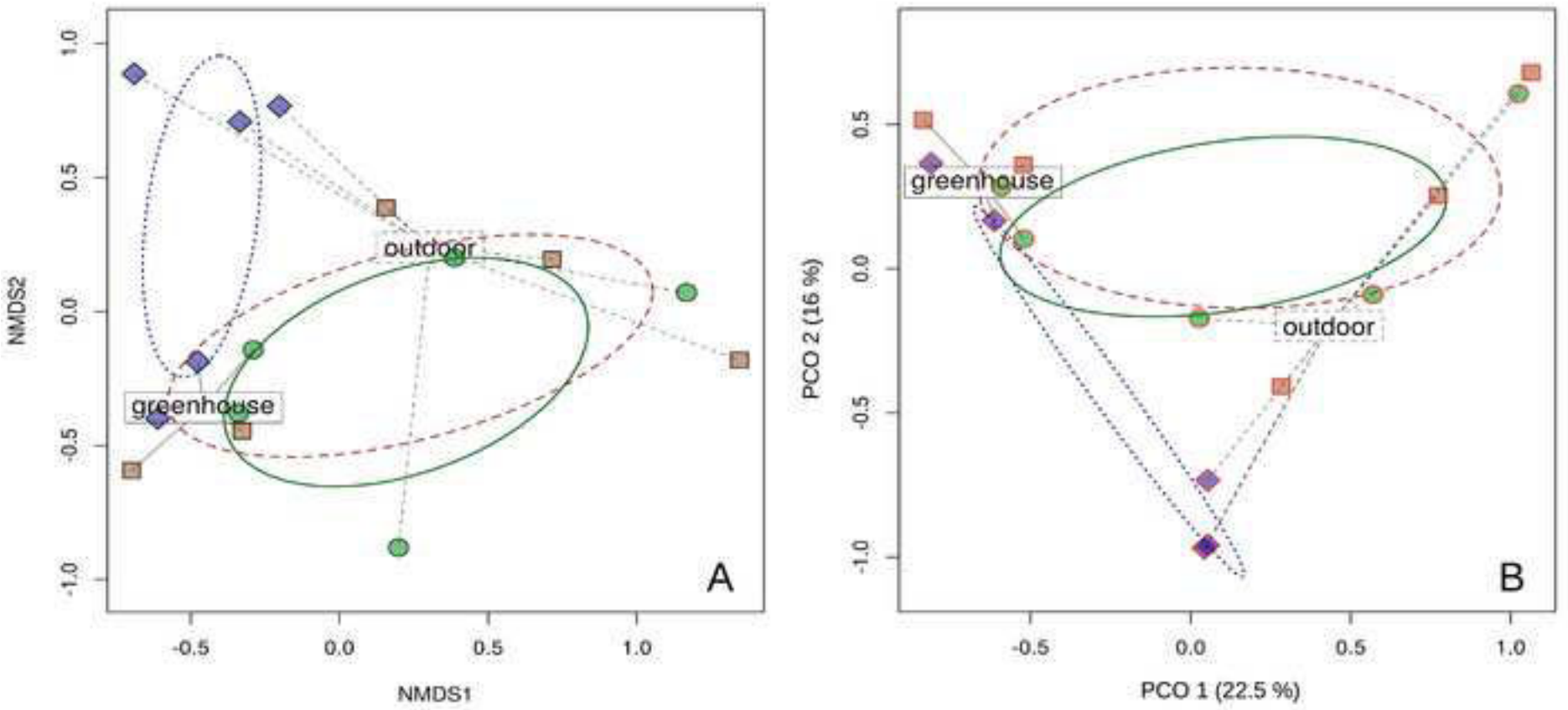

Preliminary screening for antimicrobial activities of leaf-inhabiting fungal endophytes isolated from fig tree species (*Ficus* spp.)

Michael Jay. L. Solis[1,2]*, Simon Merdivan[3], Martin Unterseher[1] & Ulrike Lindequist[3]

[1] Ernst-Moritz-Arndt University Greifswald, Institute of Botany and Landscape Ecology, Soldmannstr. 15, 17487 Greifswald, Germany

[2] Department of Natural Sciences, College of Science and Information Technology, Ateneo de Zamboanga University, La Purisima St. 7000 Zamboanga City, Philippines

[3] Ernst-Moritz-Arndt University Greifswald, Institute of Pharmacy, Friedrich-Ludwig-Jahn-Straße 15a 17487 Greifswald, Germany

*corresponding author: mjhay_2000ph@yahoo.com, tel. +49 (0)3834 / 864184, fax. +49 (0)3834 / 864114

Abstract

Fungal endophyte ecology is greatly underexplored in the Philippine forests and artificial greenhouses and therefore investigating plants from less-studied habitats could be potential sources of novel bioactive secondary metabolites. Following isolation of 2, 387 fungal endophytes from various fig tree species in different forests and greenhouses, we selected 50 isolates for preliminary screening for secondary metabolite production. We observed 14 biologically-active fungal isolates belonging to 7fungal genera, of which *Amyloporia* sp. a previously unstudied fungi for antimicrobials, was the best performing isolate. All other fungi produced also significant activities of broad and specific actions towards a range of test organisms. The results supports the presence of diversity of biologically-active fungal endophyte from different hosts species and environmental habitats.

Keywords: *Ficus*, greenhouse, bioprospecting, tropical forest, biodiversity

Introduction

While fungal pathogens induce visible alterations in plant host morphology and function, endophytes remain hidden in asymptomatic hosts (Ludwig-Müller 2015). However, fungal endophytes may also turn pathogenic or saprophytic when circumstances become unfavourable or host plants start to senescense (Aly et al. 2011).

Forest tree species host horizontally-transmitted fungal endophytes where most are filamentous ascomycetes. This plant-fungi association is believed to have begun in the early Devonian period and became a successful survival strategy among fungi (Suryanarayanan and Uma Shaanker 2015). Nevertheless, our understanding of endophytism as a lifestyle among fungi and the interaction of fungal endophytes with their plant hosts in the ecosystem has only just began. Fungal endophytes are known as indispensible mutualistic partners of higher plants that provide various benefits to their hosts such as providing stress tolerance from both biotic and abiotic factors thus allowing plants to adapt to various environmental conditions (Nagabhyru et al. 2013; Porras-Alfaro et al. 2013).

Increase interests on fungal endophytes over recent years as evident from the increase of publications are largely due to their potential for producing elaborate novel and structurally diverse bioactive secondary metabolites (Prado et al. 2013). Secondary metabolites are low molecular weight compounds not required for growth in pure culture and are produced as an adaptation for specific functions in nature (Nisa et al. 2015). These metabolites are synthesized through various metabolic pathways (eg. polyketide, isoprenoid, amino acid derivation) (Tenguria et al. 2011). The wide variety of chemicals synthesized by endophytes confer plants with more resistance to nematodes, insects and livestock while in return demanding nutrition (Strobel and Daisy 2003). The endophyte-derived compounds belong to diverse structural groups (eg. terpenoids, steroids, xanthones, chinones, phenols, isocoumarins, benzopyranones, tetralones, cytochalasines, and enniatines (Schulz et al. 2002). These are capable of exhibiting a variety of bioactivities including anti-bacterial, anti-viral, anti-fungal, anti-cancer and anti-malarial activities (Suryanarayanan and Uma Shaanker 2015). This represent a huge resource for the isolation of novel biomolecules and biocatalysts for applications in medicine, agriculture, and industry (Ally et al. 2011; Mousa and Raizada 2013).

The genus *Ficus*, collectively known as figs, is a key component of tropical forests and consists of over 800 species of trees, shrubs, vines and epiphytes in the family Moraceae. The sheer number makes the *Ficus* genus one of the most populous in number of species of all plant genera. They are widely distributed in most tropical and subtropical regions throughout the world (Berg 1989). Edible figs are prominent in both human and animal nutrition around the globe where enough warmth and moist persist to sustain their growth. Hence, it is generally agreed that figs originated in Asia and became successful in spreading around the world (Lansky and Paavilainen 2011). Figs are traditionally used as medicine or food plants, ornamental trees, religious plants, lacca hosts, fodder, fuel wood, hedges or enclosures. The importance of *Ficus* as a global spiritual and material resource for humans has been well-documented (Xu et al. 1996; Wilson et al. 2013).

The fig tree species selected for this study as sources of fungal endophytes are *F. religiosa*, *F. F. elastica* and *F. benjamina*. All 3 species are well-known medicinal plants. *Ficus religiosa*, also known as the "Bo tree" or "Bodhi tree" are employed as sources for medicines in India and throughout the Far East as antineuroinflammatory and anticonvulsant (Singh and Goel

2009); *F. elastica*, also known as the "Indian rubber tree" are known as anti-inflammatory agents and polyprenols (Hanelt et al.2001); and *F. benjamina*, also known as the "Weeping fig" are known to cure arthritis, rheumatism, used as liver medicine and as an antinociceptive (Parveen et al. 2009).

Here, all hosts where acquired from different environmental habitats (ie. natural forest, urban forest, indoor botanical greenhouse). It is interesting to determine whether the type of growth condition has any influence on secondary metabolite production. In this study, we initiated a bioprospecting effort by preliminary assessing the potential of 50 foliar fungal endophyte strains isolated from *Ficus* host species (*F. religiosa*, *F. elastica*, *F. benjamina*) and aimed to determine antimicrobial activities of the selected fungal isolates against various test bacteria and fungi.

Materials and methods

2.1 Host species

With approximately 750 distinguished species, the monoecious genus *Ficus* belong to a comparatively large group of vascular plants of tropical and subtropical distribution. The three investigated *Ficus* hosts *F. benjamina*, *F. elastica* and *F. religiosa* are naturally distributed within mostly tropical and a few subtropical Asia-Pacific regions (eg. Nepal, Bhutan, India, Indonesia, China, Philippines, or tropical Australia). Furthermore, these species are widely cultivated ornamental plants in tropical greenhouses. *Ficus benjamina* is one of the most common office and indoor plants outside the tropics although it is known to cause hypersensitivity reactions of people exposed to latex and other allergens of this plant (Delbourg et al. 1995) . *Ficus* leaves are distinctly evergreen; however habit and morphology (e.g. thickness and leaf dimensions) differ among species. *Ficus elastica* (thick, oblong leaves, 35 × 17 cm) and *F. religiosa* (broadly ovate to ovateorbiculate leaves, 7 × 4 cm) typically grow up to 30 m high while *F. benjamina* (oblong, elliptic, lanceolate, or ovate leaves, 4 × 1.5 cm) typically reaches heights of approx. 10 m.

2.2 Host collection sites

The collection sites in Germany are located in the tropical greenhouses of the botanical gardens of Berlin and Greifswald. Both sites are 250 km apart and are considered as two independent replicates. Outdoor collection sites included Mt. Makiling (eastern slope), Los Baños, Laguna; Mt. Palay-Palay/Mataas na Gulod National Park, Ternate, Cavite; and Manila City. Mount Makiling (4224 ha, elevation 1109 m; 65 km south of Manila City) and Mt. Palay-Palay/Mataas na Gulod National Park (4000 ha, elevation 648 m; 60 km southeast of Manila and 60 km northwest of Mt. Makiling) is comprised of primary and secondary forests (Solis et al. 2015). Manila City (3354 ha elevation 16 m) is a highly urbanized and industrialized area with patches of urban forests and gardens. Given the distance of around 40 km among the three sites, they too are considered independent replicates within this investigation area.

2.3 Field work and sampling processing

Collection and processing of the leaves followed identical protocols for both greenhouse and outdoor samples. Sampling was done from November 2012 to March 2013. Collection sites

located within short-distances to the laboratory were transported in sterile bags inside coolers and processed immediately, otherwise leaf samples were stored at 4°C and processed within 12 h (Solis et al. 2015a, 2015b). In brief, healthy and mature leaves (largest leaves in an adult tree ca. 10–15 m high) with no visible morphological abnormalities (ie. fungal growth, necrosis, pigmentation loss, physical damage) were collected randomly and further processed in the laboratories within 24 h. The leaves were thoroughly rinsed with sterile distilled water to remove any dirt and debris and surface sterilized by submerging into 70% ethanol for 2 min, sodium hypochloride (1% active chloride) for 5 min, and 70% ethanol for 1 min respectively. Four plugs per leaf with 10 mm diameter were produced with a sterile cork borer under sterile conditions. One hundred leaf plugs per tree species and site were further homogenized according to the dilution-to-extinction protocol for foliar endophytes (Unterseher and Schnittler 2009) and plated onto sterile 48-well multiwell plates (Carl Roth, Karlsruhe, Germany) containing 1.5 % Malt Extract Agar (MEA)supplemented with Tetracycline (10 mg l^{-1}) and Cyclosporine (10 µl l^{-1}). The plates were examined regularly for 30 days and emerging fungal colonies were axenically transferred onto fresh MEA plates for morphological analysis, morphotype grouping and DNA extraction. Duplicates of the Philippine cultures were shipped to Greifswald following a material transfer agreement between the University of Santo Tomas, Manila and the Ernst-Moritz-Arndt University of Greifswald. Vouchers are deposited in both Universities and in parts in the German Collection of Microorganisms and Cell Cultures (DSMZ).

2.4 DNA extraction, amplification and sequencing

Fungal genomic DNA was extracted using common CTAB protocols and the commercially available Master Pure Yeast DNA Purification Kit (epicentre, Madison, U.S.A). Amplification of the internal transcribed spacer (ITS) region was performed with primers ITS1 and ITS4 (White et al. 1990) and chemicals from the MangoTaq DNA Polymerase kit (Bioline, London, UK) (15.64 µl ddH_20, 5 µl Mango-Buffer, 1.7 µl $MgCl_2$ (50 mM), 0.5 µl dNTP (10 mM), 0.5 µl of each primer (10 pM/ µl), 0.16 µl (5 U/ µl) of Taq DNA polymerase and 1 µl template DNA; (5 min at 94°C, followed by 35 cycles (35 s at 94°C, 50 s at 52°C, 1 min 30 s at 72°C), and 5 min at 72°C. PCR products were run on agarose gel (0.8%) for control. The unpurified amplicons were shipped to Beckman Coulter Genomics (Takeley, UK) for sequencing. Chromatograms were manually inspected to eliminate erroneous sequences. and remnants of the flanking 18S and 28S ribosomal RNA gene were removed with ITSx (Bengtsson-Palme et al. 2013). Taxon assignments and other basic data are provided below and as online supporting information.

2.5 OTU clustering

The ITS1 region was used for OTU clustering due to its higher sequences variability and higher efficiency for species discrimination compared with ITS2 (Wang et al. 2015). Qiime's "pick_otus.py" function was used with default settings at 90, 95, 97 and 98.5 % sequence similarity thresholds. Sequence processing continued with extraction of representative sequences, taxon annotation with the UNITE reference data base and computing of final OTU tables. In order to decide about the most appropriate OTU threshold for taxonomic assignment of the filamentous fungi, we compared the different OTU groupings with morphotype information, ITS phylogeny and BLAST searches.

2.6 Fermentation and extraction of crude extracts

A total of 50 fungal endophyte isolates were selected for the preliminary screening of antimicrobial activities. Fungi was cultured in 100-mL Erlenmeyer flasks (done in triplicates), each containing 60 mL Malt extract broth (MEB, 180 ml in total), and incubated for 30 d. After filtration, fermentation broth and mycelia were lyophilized. The dried media extract were stored for antimicrobial assay. The mycelia was extracted using ethyl acetate (EtOAc) of equal volume, filtered, evaporated to dryness and stored for antimicrobial assay.

2.7 Antimicrobial Assay

Antimicrobial assays were performed in vitro using the disk diffusion method following the procedure of Gaudreau and Gilbert (1997) against *Bacillus subtilis, Staphylococcus aureus, Eschericia coli, Pseudomonas aeruginosa, Candida albicans*. The assay was performed in triplicates, and results were expressed as mean ± SD. Statistic analysis was carried out using a T-test to compare means between crude extracts and control groups to determine significant differences.

3. Results

Antibacterial activities of endophytic fungal extracts

A total of 2,387 fungal endophyte isolates of three *Ficus* host species (*F. religiosa, F. benjamina,* and *F. elastica*) were recovered from the 5 collection sites (Berlin greenhouse, Greifswald greenhouse, Mt. Makiling, Mt. Palay-Palay, and Manila). Among all the endophyte isolated in this study, a total of 50 fungal strains were pre-selected for bioprospecting. This selection pool of different fungal strains was designed to represented all collection sites and host sources. From these samples, the fermentation broth/media and mycelial crude extracts from a total of 14 fungal isolates displayed significant antibacterial activities against one or more test organisms (Table 1). These activities are shown as bacteria-free circular clearings or zones of inhibition (ZOB) (Figure 1) against gram-positive (ie. *Staphylococcus aureus, Bacillus subtilis*) and gram-negative bacteria (*Escherichia coli*).
Crude extracts from 7 isolates (ie. *Acremonium* sp., *Amyloporia* sp., *Colletotrichum* sp.1, *Colletotrichum* sp.2, *Mycosphaerella* sp.1, *Sordariomycete* sp.4, *Talaromyces* sp.) exhibited significant activity specifically against *S. aureus* only while one isolate (ie. *Phomopsis* sp.2) showed activity only for *B. subtilis*. There were also isolates that was specifically positive for all gram-positive test bacteria (ie. *Phomopsis* sp.1, *Sordariomycete* sp.1, *Sordariomycete* sp.2, *Sordariomycete* sp.3). Broad-spectrum activity against both gram-negative and gram positive bacteria were also detected from 3 isolates (ie. *Mycosphaerella* sp.2, *Phomopsis* sp.2, *Talaromyces* sp.). Mycelial crude extracts of 6 isolates (ie. *Amyloporia* sp., *Phomopsis* sp.3, *Sordariomycete* sp.1, *Sordariomycete* sp.2, *Sordariomycete* sp.3, *Sordariomycete* sp.4) were also active against *S. aureus* and *B. subtilis*. All fungal isolated were also tested against the gram-negative bacteria *Pseudomonas aeruginosa* and the fungi *Candida albicans*, however no activity were detected.

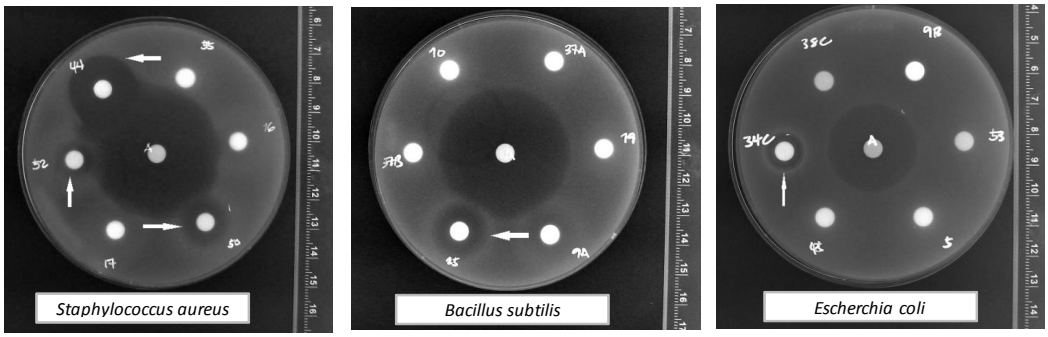

Figure 1. Antibacterial activities of fungal endophytes shown in culture plates measured as zone of inhibition (mm).

Table 1 The antibacterial activities of extra- and intra-cellular metabolites of the crude extracts from *Ficus* fungal endophyte species shown as zones of inhibition (mm).

	Zone of Inhibition (mm)					
	Staphylococcus aureus		***Bacillus subtilis***		***Escherichia coli***	
Taxon (host/site)	**Media extract**	**Mycelia extract**	**Media extract**	**Mycelia extract**	**Media extract**	**Mycelia extract**
Acremonium sp. (Fe/Berlin greenhouse)	16.0* ± 1.0	–	–	–	–	–
Amyloporia sp. (Fb/Greifswald greenhouse)	22.7* ± 1.2	20.0* ± 5.7	–	–	–	–
Colletotrichum sp.1 (Fb/Mt. Makiling)	11.0* ± 0.0	–	–	–	–	–
Colletotrichum sp.2 (Fe/Mt. Makiling)	11.0* ± 0.0	–	–	–	–	–
Mycosphaerella sp.1 (Fr/Berlin greenhouse)	10.7* ± 1.5	–	–	–	–	–
Mycosphaerella sp.2 (Fb/Berlin greenhouse)	13.7* ± 1.5	–	–	–	14.7* ± 1.2	–
Phomopsis sp.1 (Fe/Manila urban forest)	14.7* ± 0.6	–	13.7* ± 0.6	–	–	–
Phomopsis sp.2 (Fe/Manila urban forest)	16.3* ± 0.6	–	–	–	13.7* ± 0.6	–
Phomopsis sp.3 (Fb/Manila urban forest)	–	–	13.3* ± 0.6	16.0* ± 0.0	–	–
Sordariomycete sp.1 (Fe/Berlin greenhouse)	12.3* ± 1.5	12.0* ± 0.0	12.0* ± 2.0	12.0* ± 0.0	–	–
Sordariomycete sp.2 (Fe/Mt. Makiling)	13.0* ± 1.0	10.0* ± 0.0	11.3* ± 1.5	12.0* ± 0.0	–	–
Sordariomycete sp.3 (Fb/Mt. Makiling)	12.6* ± 2.4	13.0* ± 1.4	11.0* ± 1.0	14.0* ± 0.0	–	–
Sordariomycete sp.4 (Fe/Berlin greenhouse)	11.3* ± 0.6	12.0* ± 2.8	–	–	–	–
Talaromyces sp. (Fe/Mt. Makiling)	12.7* ± 0.3	–	–	–	–	–
Amphicillin	39.0* ± 2.7	42 ± 0.0	28.4 ± 1.7	25.6 ± 0.5	29.0 ± 1.0	–

**** Significant (P≤0.05)*** **; ± *standard deviationof three replicates;* – *no inhibition***

4. Discussion

The preliminary antimicrobial screening revealed multiple and diverse fungal species from different host species and various types of environments producing strong and significant bioactive activities. This only further support the popularity and recognition of fungal endophytes as rich and untapped sources of valuable secondary metabolites.

In our study, we noticed that some bioactive fungal species were only isolated from specific environments while similar species isolated from other environments produced negative activities. For example, all bioactive *Phomopsis* spp. were only detected in the urban forest (Manila) and all other *Phomopsis* spp. in natural forests and greenhouses tested negative. Similarly, *Mycosphaerella* spp. were isolated from the urban forest however those only isolated in natural forests were positive for bioactivity. In the case of *Phomopsis* spp., this may indicate that pollutants present in its growth environment may have influenced the production of secondary metabolites. This environmental influence was observed by Dreyfuss et al. (1994) where the production of chemicals (ie. cyclosporin A, enchinocandin B, papulacandins and verrucarins) varied depending on the habitat and substrate the fungi grows and adapted to. In our study, the respective habitat or ecological niche may have influenced the fungi to enhanced their synthesis of bioactive metabolites (Tenguria et al. 2011).

Among the 14 bioactive fungal isolates, the most promising was *Amylopria* sp. isolated exclusively from the Berlin greenhouse. This wood rot-fungi, previously described in China, North and South America, and Europe (Cui and Dai 2013; Ortiz-Santana et al. 2013) produced the strongest activity but has not yet been analyzed for secondary metabolites. The potential of this species as source of novel chemicals is promising and merits deeper chemical analysis. *Phomopsis* spp. were the most versatile species with broad-spectrum as well as specific activities against test bacteria. This is not surprising as these species have been among strongest bioactive fungi surveyed (Buatong et al. 2011). Despite many secondary metabolite investigation done on *Phomopsis* species over the past 2 decades, new secondary metabolites have been continuously discovered from this fungi (Cheng et al. 2015; Huang et al. 2015). In fact, metabolites in *Phomopsis* and related Diaporthales may be species-specific, giving support to the use of metabolite profiling and chemical classification for phenotypic recognition and delimitation of species (Abreu et al. 2012).

The leaves of the 3 host *Ficus* species (ie. *F. religiosa*, *F. benjamina*, *F. elastica*) are well-known in literature as medicinal plants. In general, the leaves of fruit trees contain stronger antioxidants than the fruits. Fruit tree leaves are less in the edible class than are fruits, and are closer to medicines and the higher degree of medicality is associated with higher levels of toxicity (Lansky and Paavilainen 2011). These figs had been used directly for the isolation and characterization of bioactive metabolites, however medicinal plants are also recognized as a repository of biologically active fungal endophytes that are pharmaceutical important (Tejesvi et al. 2007). The chemical metabolites present in the leaves of host species may also have an influence on fungal secondary metabolite production. Ludwig and Muller (2015) suggested that host plant and/or endophyte metabolism can be induced by the other partner and that partial or complete biosynthesis pathways for plant secondary metabolites can be attributed to such endophytes. In other cases the host plant is able to metabolize substances from fungal origin. It has been reported that fungal endophytes might synthesize metabolites similar to or even more active than those produced by their hosts (Strobel 2002).

In our study, we observed that all bioactive fungi where capable of producing extracellular metabolite (media/fermentation crude extract) while only some produced intracellular metabolites (mycelial crude extract). Extracellular metabolites sometimes are more effective than intracellular metabolites (Soltani et al 2014) but our results show almost equal activity. The production of extracellular metabolites of our isolates suggest that these chemicals may be used by the fungi to attack or compete with other species or cells in mixed populated ecological niches (Romeralo et al. 2015) .

Only 14 from the total of 50 tested for the preliminary screening were positive for antimicrobial activity. This relatively low number of positive isolates may be a result of methodological reasons. In all the tests, we only used one type of media (Malt extract broth) and could be the reason behind the absence of a biological activity. The type of endophyte-pathogen interaction might be medium-specific (Prada et al. 2009) or the culture media used may either lack or contain excessive nutritional requirements (Tong et al. 2011; Miles et al. 2012). However, such variation might also be accounted for by the instability of the metabolites, a volatile nature of the substances produced, or a poor diffusing capacity into the agar medium (Miles et al. 2012). In addition, media composition, temperature, pH, culture vessel, aeration, cultivation time, light intensity can increase or reduce the production of the bioactive compounds by the fungal strain (Bode et al. 2002; Bills et al. 2008; Siqueria et al. 2011). As a preliminary test, we did not use a significant experimental design to control all these factors that may lead to erroneous results or results that cannot be replicated.

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4. Conclusion and Perspectives

While the current status of Philippine fungal endophyte ecology remains at infancy, our research work has generated meaningful ecological data on Philippine fungal endophytes associated with one of the most diverse tree species in the tropics. Certainly, this is a positive stride towards building a model for future local ecological surveys in the hope of accumulating a robust Philippine fungal literature database. Although previous research studies on fungal endophyte biodiversity in the country are available, ecological knowledge of local endomycobiota are far too scarce and fragmented. As a megadiversity hotspot, the potential of ecological data awaiting to be discovered are far too promising to be continuously ignored especially in the backdrop of persistent threat of habitat loss and species extinction.

To achieve this goal, a credible and meaningful fungal research agenda is needed, therefore the primary goal at the end of this PhD study is to form a specialized working group in my home university dedicated solely for the pursuit of fungal endophyte biodiversity research in the Philippines. However, this scientific endeavour comes without challenges. As a developing economy, the scientific community is lacking in available research funds and also suffers from poor research infrastructure from decades of neglect deep rooted from the lack of priority for research. Throughout the conduct of this 3-year research project, strategic international and local links with accomplished (Prof. Dr. Marc Stadler, Prof. Dr. Ulrike Lindequist, Dr. Andrey Yurkov, Dr. Thomas dela Cruz) and promising young scientists, as well as international organizations (German Mycological Society) were established to open the doors for future research collaborations. This, in addition to the already well-established working relations with scientists (Dr. Martin Unterseher and Prof. Dr. Martin Schnittler) in our own working group.

The fostering of research cooperation are essential in designing and presenting robust and substantial research proposals in boosting the chances of successfully acquiring research grants from international and local funding institutions and agencies. Furthermore, this would facilitate the exchange of scientific expertise and technology that would greatly be beneficial in enhancing and advancing the scientific capabilities of local scientists in the Philippines. For example, a proposed future project with Dr. Martin Unterseher for the implementation of next-generation technology in generating local fungal endophyte NGS data for highly endemic plant species and endangered habitats.

The desired impacts being aimed as a result of this PhD endeavour is not only directed towards generating serious fungal studies but also in the context of generating novel scientific knowledge as valuable resource material for university teaching in upgrading the quality of student education. Furthermore, it is also an important future aspiration to share scientific knowledge to draw the attention of various sectors (scientists, conservationists, volunteer groups, environmental media, national legislature) involved in Philippine biodiversity conservation to previously overlooked biodiversity-rich areas for designation as protected areas, and more importantly to harmonize and clarify policies and resolve inconsistencies in national conservation programs.

DECLARATION

Eigenständigkeitserklärung

Hiermit erkläre ich, dass diese Arbeit bisher von mir weder an der Mathematisch-Naturwissenschaftlichen Fakultät der Ernst-Moritz-Arndt-Universität Greifswald noch einer anderen wissenschaftlichen Einrichtung zum Zwecke der Promotion eingereicht wurde.
Ferner erkläre ich, dass ich diese Arbeit selbstständig verfasst und keine anderen als die darin angegebenen Hilfsmittel und Hilfen benutzt und keine Textabschnitte eines Dritten ohne Kennzeichnung übernommen habe.

Michael Jay L. Solis

Erklärung bei Gemeinschaftarbeiten

Hiermit erkläre ich, dass die in der folgenden Inhaltsübersicht mit meinem Namen gekennzeichneten Kapitel von mir selbständig verfasst worden sind:

1	Abstract	Solis MJL
2	Introduction	Solis MJL
3	Publications	
3.1	Leaf-inhabiting endophytic yeasts are abundant but unevenly distributed in three *Ficus* species from botanical garden greenhouses in Germany	Solis MJL, Yurkov A, dela Cruz TE, Unterseher M
3.2	The diverse community of leaf-inhabiting fungal endophytes from Philippine natural forests reflects phylogenetic patterns of their host plant species *Ficus benjamina, F elastica* and *F religiosa*	Solis MJL, dela Cruz TE Schnittler M, Unterseher M
3.3	Diverse and distinct leaf-inhabiting endophytic communities were revealed during cultivation studies with three *Ficus* species from artificial greenhouses and natural 2 outdoor conditions	Solis MJL, dela Cruz TE Unterseher M
3.4	Preliminary screening for antimicrobial activities of leaf-inhabiting fungal endophytes from fig tree species (*Ficus* spp.).	Solis MJL, Merdivan S, Unterseher M, Lindequist U
4	Conclusion and Perspective	Solis MJL

3.1 Solis MJL, Yurkov A, dela Cruz TE, Unterseher M. 2015. Leaf-inhabiting endophytic yeasts are abundant but unevenly distributed in three *Ficus* species from botanical garden greenhouses in Germany. *Mycological Progress.* 14:1019. (http://dx.doi.org/10.1007/s11557-014-1019-6).

MJLS and MU designed the study. MJLS and MU jointly carried out all field and lab work (isolation of fungal endophytes), molecular work (DNA extraction, PCR). Biodiversity and phylogenetic analysis were jointly done by MJLS and MU. AY provided own in-house ITS database for *Cryptococcus* and *Rhodotorula* species as reference sequences. The manuscript was primarily written by MJLS with significant contributions from MU, AY and TEDC.

3.2 Solis MJL, dela Cruz TE, Schnittler M, Unterseher M. 2016. The diverse community of leaf-inhabiting fungal endophytes from Philippine natural forests reflects phylogenetic patterns of their host plant species *Ficus benjamina*, *F. elastica* and *F. religiosa*. *Mycoscience*. (http://dx.doi.org/10.1016/j.myc.2015.10.002).

MJLS and MU designed the study. MJLS performed all field and lab work (isolation of fungal endophytes), molecular work (DNA extraction, PCR). Data management, biodiversity and phylogenetic analysis were jointly done by MJLS and MU. The manuscript was primarily written by MJLS with significant contributions from MU, TEDC and MS.

3.3 Solis MJL, dela Cruz TE, Unterseher M. submitted. Cultivated leaf-inhabiting endophytic fungi from fig tree species (*Ficus* spp.) in German tropical greenhouses form distinct communities compared with natural outdoor conditions of the Philippines. *Biodiversity and Conservation* .

MJLS and MU designed the study. MJLS and MU jointly carried out all field and lab work (isolation of fungal endophytes), molecular work (DNA extraction, PCR). Data management, biodiversity and phylogenetic analysis were jointly done by MJLS and MU. The manuscript was primarily written by MJLS with significant contributions from MU and TEDC.

3.4 Solis, MJLS, Merdivan S, Unterseher M, Lindequist U. in preparation. Preliminary screening for antimicrobial of leaf-inhabiting fungal endophytes from fig tree species (*Ficus* spp.).

All authors contributed equally in designing this study. MJLS conducted the lab work with assistance from MU and SM on technical lab details. UL is the senior adviser on all scientific work. The manuscript was primarily written by MJLS with significant contributions from all co-authors.

***

All R-scripts used in the biodiversity and statistical analysis were developed by MU and are provided with the publications as supplementary files. Permanent cultures of representative isolates were deposited at the DSMZ—German Collection of Microorganisms and Cell Cultures, Braunschweig. All ITS sequence data were submitted to the European Nucleotide Archive (ENA). All primary data are made available as DVD, which is attached to the thesis.

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Michael Jay L. Solis
(Author of the thesis)

Die Unterschrift weiterer Autoren kann aus technischen Gründen nicht eingeholt werden. Da die betreffenden Kapitel jedoch in Zeitschriften veröffentlicht sind, haben alle Mitautoren in die Publikation eingewilligt.

PD. Dr. Martin Unterseher
(Wissenschaftlicher Betreuer)

Erklärung zur Agabe einer elektronisher Kopie der Dissertation

Mathematisch-Naturwissenschaftliche Fakultät
Einverständniserklärung nach § 4 Abs. 1 Nr. c Promotionsordnung

Hiermit erkläre ich, dass von der Arbeit eine elektronische Kopie gefertigt und gespeichert werden darf, um unter Beachtung der datenschutzrechtlichen Vorschriften eine elektronische Überprüfung der Einhaltung der wissenschaftlichen Standards zu ermöglichen.

Datum: 15.04.2016

Unterschrift: Michael Jay L. Solis

CURRICULUM VITAE

PERSONAL INFORMATION

Name **SOLIS, MICHAEL JAY L.**

Address Hans-Beimler Straße, D-17491 Greifswald, Germany
Telephone +49-17671977809
E-Mail mjhay_2000ph@yahoo.com
Date of birth 30 September 1976 in Bacolod City, Philippines

EDUCATION

2012 – 2016 **Ernst-Moritz-Arndt University Greifswald, Germany**
Department of Botany and Landscape Ecology
Doctorate degree (Dr. rer. nat.)

2005 – 2009 **University of Santo Tomas, Manila Philippines**
Masters of Science in Microbiology

1993 – 1998 **University of the Philippines Los Banos, Philippines**
Bachelor of Science in Biology (Major in Cell Biology)

PROFESSIONAL EXPERIENCE

2002 – Present **Ateneo de Zamboanga University Zambonga City, Philippines**
Department of Natural Sciences, College of Science and Information Technology
(Tenured faculty member)

1999-2000 **Western Mindanao State University Zamboanga City, Phlippines**
Department of Biology, College of Science and Math
(Faculty member)

PERSONAL SKILLS AND COMPETENCES

Mother tongue Filipino
Other languages English (excellent reading, writing, and verbal skills)
German (basic skills)

Greifswald _____ _____
Date 15.04.2016 Michael Jay L. Solis

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