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DATES:
Received: 25 Nov. 2019
Revised: 28 May 2020
Accepted: 17 Aug. 2020
Published: 29 Mar. 2021

HOW TO CITE:
Kemler M, Wingfield MJ, Cowan DA, Slippers B. Foliar fungi of the enigmatic desert plant *Welwitschia mirabilis* show little adaptation to their unique host plant. S Afr J Sci. 2021;117(3/4), Art. #7666. https://doi.org/10.17159/sajs.2021/7666

ARTICLE INCLUDES:
☒ Peer review
☒ Supplementary material

DATA AVAILABILITY:
☒ Open data sets*
☐ All data included
☐ On request from author(s)
☐ Not available
☐ Not applicable
**see accession numbers in text*

EDITORS:
Teresa Coutinho
Salmina Mokgehle

KEYWORDS:
Welwitschia mirabilis, Namib Desert, foliar fungi, *Alternaria*, *Aureobasidium*

FUNDING:
Department of Science and Innovation (DSI)–National Research Foundation (NRF) Centre of Excellence in Tree Health Biotechnology (CTHB), University of Pretoria

Foliar fungi of the enigmatic desert plant *Welwitschia mirabilis* show little adaptation to their unique host plant

Foliar fungi, especially endophytic fungi, constitute an important part of the microbiome of plants. Yet little is known about the composition of these communities. In this study, we isolated fungi from leaf tissues of the desert plant *Welwitschia mirabilis* to determine the culturable diversity of the foliar fungal community. The isolated fungal taxa, which grouped into 17 distinct lineages, were identified by sequencing elongation factor 1 alpha, beta-tubulin 1, beta-tubulin 2 and the internal transcribed spacer region. The culturable community was mainly composed of cosmopolitan fungal genera despite the unique taxonomic position of the plant and its geographic isolation. To test for endemism in two of the common fungal genera, *Alternaria* and *Aureobasidium*, we built haplotype networks using a global data set. Even this broad data set showed little evidence for specialisation within this unique host or its geographical location. The data suggest that the culturable members of communities of leaf-associated fungi in habitats with little plant coverage, such as the Namib Desert, are mainly established by long-distance aerially distributed fungal inocula and few of these taxa co-evolve with the host within the habitat.

Significance:

- The culturable members of fungal communities associated with an ecological and evolutionary isolated plant have not co-speciated with their hosts, but to a large extent are composed of globally distributed fungal species.
- Harsh environmental conditions and the geographic isolation of host plants seem to favour ubiquitous fungal species over more specialist fungal species.

Introduction

Fungi and plants have a long history of co-evolution and plant–fungal interactions are thought to have been essential in the establishment of plants in terrestrial environments. For example, fossil records of interaction structures of arbuscular mycorrhizal fungi and Embryophyta have been recovered from the Rhynie cherts and date back to more than 400 Ma[1], possibly as old as 475 Ma[2]. Structures of endophytic fungi (i.e. microorganisms that in part or during their whole life cycle colonise plant tissues without visible symptoms[3]) in prostrate axes have been described from as early as 400 Ma.[4] Similarly, leaf-associated endophytes are known from the 300 Ma Carboniferous era.[5] These studies show that, despite the unknown interaction type between host and fungus, aboveground organs of plants, the so-called phyllosphere, have served as a suitable habitat for fungi for a long time.

Extant phyllosphere-associated fungi including endophytes and epiphytes (i.e. fungi that grow on the surface of plants) mostly show no visible impact on their host under favourable conditions or even enhance plant performance. Some studies have provided evidence that these fungi affect plant physiology. Some epiphytic and endophytic yeasts have been shown to promote plant growth[6,7], whereas some endophytes become pathogenic when the host plant experiences abiotic or biotic stress[8,9]. Despite our limited knowledge of their function, culture-dependent and culture-independent methods have identified species-rich fungal communities in the phyllosphere that add substantially to the hyperdiversity of fungi.[10-14] How these communities become established and the factors that influence them are understood only for a limited number of plant–fungus systems. In the case of endophytes, the greatest impact on the structure of communities results from geographic location and host plant.[15] It is also thought that the evolutionary history of the host and the climate[16] play additional roles. Most likely similar mechanisms apply for epiphytes.[17,18]

Plant species in deserts harbour a greater proportion of fungal endophytes with cosmopolitan distribution than the same plant species under less extreme conditions.[15] This observation is interesting in the light of two aspects. Firstly, many plant–fungus interactions are characterised by local adaptation to the host population[19,20], which is reflected in the genetic sub-structuring of the fungal population[21]. Secondly, many fungi show clear biogeographic patterns when an appropriate species concept that incorporates molecular data is applied.[22] These findings imply that geographic structure should exist in the distribution of cosmopolitan phyllosphere fungi. However, especially culture-dependent studies have recovered many fungal species that seem not necessarily restricted to plant interactions and that have been found in other habitats as well.[17,23,24] Such low host association could counterbalance restrictions to gene flow and lead to low genetic population structure. However, to the best of our knowledge, no studies of phyllosphere-associated fungi have focused on the possibility of biogeographic patterns of cosmopolitan species.

The Namib Desert is one of the oldest and driest deserts on the planet.[25] Like all desert ecosystems, it imposes severe constraints on living organisms and is characterised by the stochasticity of nutrient and water supply, as well as continuous varying UV radiation and extreme temperature changes.[26] The coastal (western) Namib Desert is unusual in that it experiences frequent fog events with a decreasing gradient from the coast towards

the Great Escarpment. It also experiences infrequent and sparse rainfall events with a decreasing gradient from the Great Escarpment towards the coast.[25]

Welwitschia mirabilis Hook, the only living member of the family Welwitschiaceae, is a unique dioecious plant that occurs natively only in the Namib Desert and adjacent savannah ecosystems in Namibia and Angola (Figure 1).[27] It is part of an old lineage of land plants, the Gnetophyta, that diverged from the Pinaceae between 121.8 Ma and 309.5 Ma ago.[28] Welwitschiaceae fossils have been found from the Lower Cretaceous, 112–114 Ma ago.[29] Although these long-lived plants (estimates reach over 2000 years[27]) have been studied extensively[30], little is known regarding their associated mycobiome. A few studies have concentrated on the effect of *Aspergillus* on seed mortality[31,32] and the level of mycorrhizal interactions[33], but to date the diversity of phyllosphere fungi associated with *Welwitschia* is unknown.

The uniqueness of the phylogenetic position of *Welwitschia*, as well as its remote habitat in one of the oldest and hottest deserts, provides an ideal opportunity to compare factors that might influence the composition of its fungal communities. As a first goal we isolated fungi to assess the diversity and uniqueness of potential endophytes colonising *Welwitschia* plants at a species level. The second goal was to consider the biogeographic structure of intraspecific diversity for some of the common cosmopolitan fungi that were isolated.

Materials and methods

Samples from *Welwitischia mirabilis* were recovered under the auspices of the Namibian Ministry of Environment and Tourism permit, number 1522/2011. Sections of leaves (about 20 cm^2) were obtained from two individuals in the Welwitschia Wash near Gobabeb (23°36.54' S; 15°10.07' E) in the Central Namib Desert in May 2011. Only leaf tissue from young sections near the stem could be obtained, as the plants showed symptoms of intense grazing and lacked any old growth leaf parts. Leaves were washed with ddH_2O and subsequently shaken for 1 h in phosphate-buffered saline[34] to remove superficially attached spores. Leaves were not surface sterilised, as our interest was in all fungi associated with this desert plant, including endophytic and epiphytic fungi. The leaves were cut into pieces of approximately 4 x 4 mm and plated on malt extract agar at room temperature (~22 °C). Emerging mycelia were transferred to fresh malt extract agar media to obtain pure cultures.

DNA from pure fungal cultures was isolated using either a salt extraction method described by Aljanabi and Martinez[35] or the Qiagen DNeasy® Plant Mini Kit (Qiagen, Hilden, Germany). The fungal barcode region, namely the rDNA *ITS*, as well as parts of elongation factor 1 alpha (*EF1a*), beta-tubulin 1 (*BTUB1*) and beta-tubulin 2 (*BTUB2*) genes for selected samples were amplified (Supplementary table 1). The *ITS* rRNA gene region was amplified using ITS1F[36] and ITS4[37]; *EF1a* was amplified using EF1f and EF1r; *BTUB1* was amplified using BT1a and BT1b[38], and *BTUB2* was amplified using BT2a and BT2b[38]. Amplicons were sequenced using the Big Dye™ Terminator Cycle Sequencing Kit V3.1 (Applied Biosystems, Foster City, CA, USA) on an ABI PRISM® 3100 Genetic Analyser (Applied Biosystems, Foster City, CA, USA).

Sequences were checked for quality, trimmed and, where applicable, a consensus sequence was built from forward and reverse strands using CLC Genomics Workbench v4.0.3 (CLC bio, Aarhus, Denmark). Phylogenetic analyses were used to assess the species composition. Phylogenetic trees were inferred for *ITS*, *EF1a* and *BTUB1* individually, as well as by applying a super matrix approach of a concatenated data set for these DNA regions. For the individual gene trees, sequences were compared against the NCBI nucleotide database using BLASTn[39]

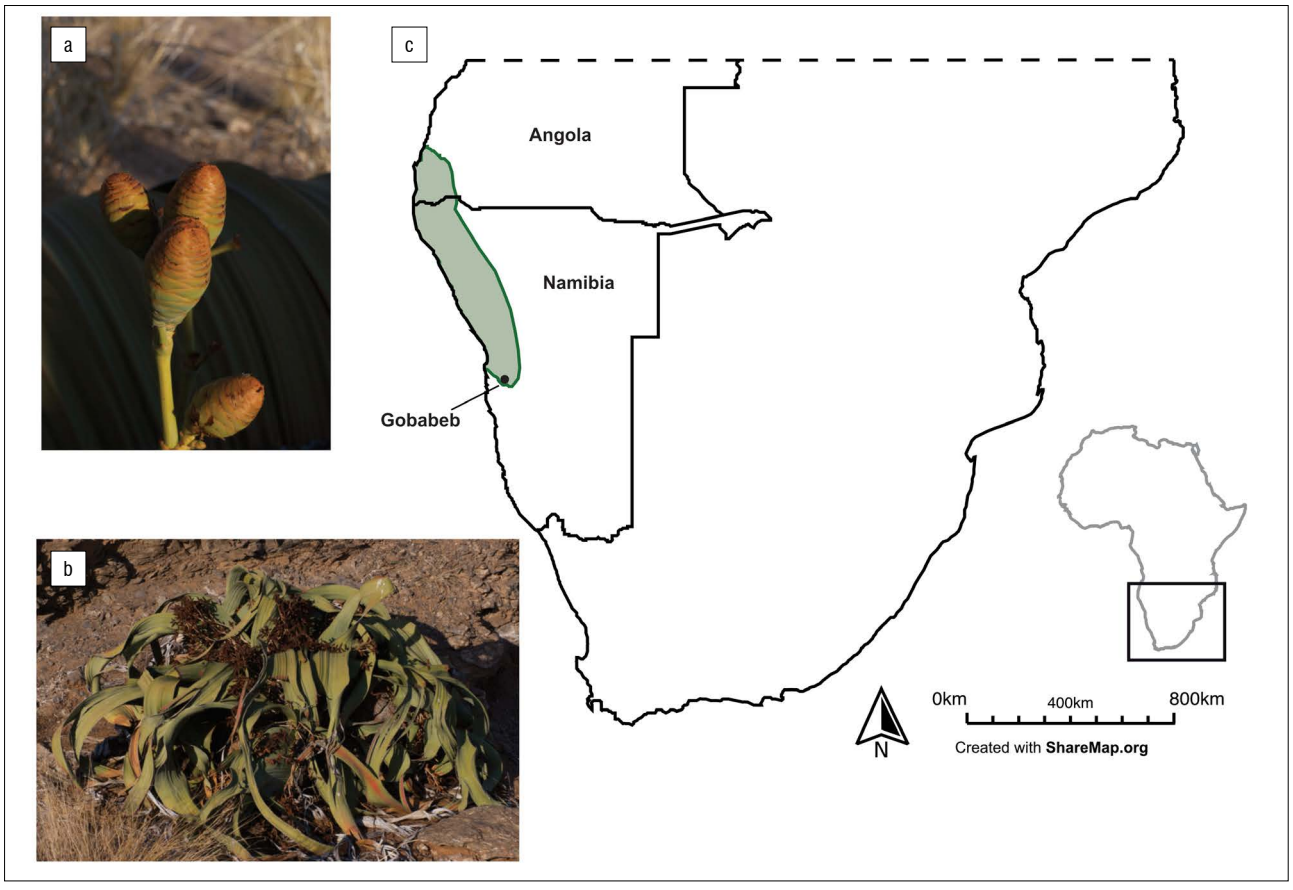

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Figure 1: (a) Female seed cones of *Welwitschia mirabilis*. (b) Male *Welwitschia mirabilis* with pollen cones. (c) Geographic distribution of *Welwitschia mirabilis* in Angola and Namibia. The sampling site near Gobabeb is indicated.

and the five hits with greatest similarity were downloaded. Alignments were generated using MAFFT v7.154b[40,41] under the auto option, which chooses the most appropriate alignment strategy for a given data set automatically. The aligned sequences were pasted into the GBlocks web interface[42] to remove ambiguous sites, thus allowing smaller final blocks and gaps in the alignment. Species names for the specimens isolated in this study were assigned based on the single tree. For the super matrix approach, selected NCBI sequences of the single gene trees that clustered close to the sequences retrieved in this study were chosen. Sequences were aligned as described for the individual genes and then concatenated using SequenceMatrix[43], whereby missing sequences were coded as missing data. Maximum likelihood phylogenies and bootstrap with 1000 replicates were inferred using RAxML 8.0.25[44] applied via the raxmlGUI[45] using the GAMMAGTR and the rapid bootstrap option.

To consider the possible effects of geographic distribution and adaptation to *W. mirabilis,* haplotype networks were reconstructed for two of the dominant fungal taxa (*Aureobasidium* and *Alternaria*) with a broader phylogenetic sampling from the NCBI database. In the case of *Aureobasidium*, individual haplotype networks were constructed for ITS and BTUB2, whereas for Alternaria they were constructed for ITS and BTUB1. After aligning the sequences in MAFFT v7.154b, the sequences were collapsed into haplotypes using Map in the SNAP workbench[46,47], thus excluding indels and infinite site violations. The pegas package[48] in R v3.0.0[49] was then used to construct minimum spanning trees from these haplotypes.

Results

The foliar fungi associated with the leaves of *W. mirabilis* that could be isolated and grown in culture belonged to either the Ascomycota or Basidiomycota (Supplementary table 1). The latter group was represented by two isolates (*Rhodotorula* and *Cryptococcus*) and these were not considered in further analyses. Within the Ascomycota, specimens belonging to the Capnodiales, Dothideales, Eurotiales, Hypocreales, Pleosporales or Xylariales were recovered and the most common taxa were *Alternaria*, *Aspergillus*, *Aureobasidium*, Didymellaceae and *Pleospora welwitschiae* s.l. Taxa with few isolates recovered belonged to *Bionectria*, *Cladosporium*, Montagnulaceae, *Penicillium*, *Pestalotiopsis*, *Stigmina* and *Talaromyces* (Figure 2). In total, 17 species or clades that might represent species were identified in the Ascomycota. The majority of taxa clustered together with fungal sequences obtained by other studies from other geographic localities. We inferred only three lineages that did not contain sequences from other studies. However, even these lineages clustered closely to sequences from fungi deposited at NCBI (Figure 2). In the Didymellaceae, we recovered one lineage that showed some genetic divergence from its sister taxon *Didymella pinodella* and another lineage that showed phylogenetic affinity to an uncultured *Phoma* sequence. One lineage clustered together with *Apioplagiostoma aceriferum* and a sequence from *Oryza sativa* (a potential fungal contamination), but with a larger genetic distance and low statistical support. Fungal specimens isolated from *Welwitschia* that fell within the genera *Alternaria* and *Aureobasidium* did not cluster according to their origin but were intermingled with sequences from other geographic origins. The sequences obtained in this study were deposited in GenBank under the accession numbers KT150524–KT150716 (Supplementary table 1).

Sequences downloaded from NCBI for the more thorough analyses of geographic clustering of the two most dominant taxa included: for *Alternaria,* 493 *ITS* and 122 *BTUB1* sequences, and for *Aureobasidium*, 376 *ITS* and 50 *BTUB2* sequences. The analysis of the *ITS* sequences for both genera inferred that the dominant haplotypes contained most of the sequences from many different geographic locations (Figure 3a and 4a). The analysis of the *BTUB1* region in *Alternaria* and the analysis of the *BTUB2* region in *Aureobasidium* showed more haplotype diversity. However, there was no clear correlation between geographic location and the occurrence of fungal groupings (Figure 3b and 4b).

Discussion

This study, although restricted to a few leaf samples because of the high protective state of *W. mirabilis*, revealed a rich fungal diversity of foliar fungi associated with the leaves of this plant. Of the 17 taxa identified, only *Pleospora welwitschiae* is a species known to be specifically associated with *W. mirabilis*. Only three other lineages, which according to our analyses could belong to as-yet unknown taxa, might potentially represent endemic or even host-specific lineages. The other taxa clustered closely with sequences of fungi from different geographic origins and appear to have widespread geographic distributions. To understand geographic sub-structuring and potential adaptation we explored haplotype patterns in more detail for the dominant cosmopolitan genera *Aureobasidium*[50] and *Alternaria*[51].

In the phylogenetic analysis, only the best matching sequences from NCBI were used for identification purposes, as is common for studies of this nature. However, low taxon sampling in phylogenetic analyses has a limited capacity to show any geographic clustering within a species. Many plant-associated fungi show geographic patterns due to local adaptations to a host population and biogeographic patterns in fungi are more prevalent than previously assumed.[22,52,53] We thus hypothesised that for adaptation to the locally restricted host plant to occur, some geographic sub-structure must exist in the common fungal taxa associated with *W. mirabilis*.

In order to identify the existence of geographic sub-structure in the two most frequently isolated fungal taxa isolated from *W. mirabilis*, haplotype networks using larger sequence data sets with publicly available data were reconstructed. Using these larger data sets, we observed no geographic clustering of our samples of *Alternaria* and *Aureobasidium* amongst global collections of closely related sequences (Figures 3 and 4). These results indicate common gene flow between these fungi on *W. mirabilis* and populations represented by downloaded sequences. They most likely also reflect frequent introduction of these fungi into the Namib Desert. Although grazers (e.g. springbok, donkeys and horses[30]) could also distribute phyllosphere fungi, given the general isolation of the environments in which *W. mirabilis* grows, with usually limited human traffic, spores of the commonly isolated and globally distributed fungi are expected to be mostly aerially dispersed. Supporting this view, studies of the biological content of aerosols have shown that fungal biomass can be up to 45% of the total weight of coarse aerosol particles and contain some of the fungi recovered in the present study.[54]

Isolation of cosmopolitan foliar fungal genera has previously been reported in other plant species[15,17,23,24], but seems even more pronounced in individuals in extreme climatic conditions[15]. Studies of endophytes from desert plants, including other gymnosperms, have shown more diverse communities than in plants of the same species from less extreme environments, but cosmopolitan taxa such as *Alternaria*, *Aspergillus*, *Aureobasidium*, *Cladosporium* and *Phoma* constituted the majority of isolated fungi.[15,55,56] In *Deschampsia antarctica*, one of the two angiosperms that grow on the Antarctic continent, endophyte diversity was generally low and the most common endophyte isolated was an *Alternaria* sp.[57] When our results are considered together with these previous studies, it appears that the pattern of high diversity but low uniqueness of plant-associated microbes might be consistent across diverse and isolated extremely dry environments (both cold and hot).

The reason for the high level of occurrence of cosmopolitan and mostly saprobic fungi in harsh environments is not known. It has, however, been hypothesised that infecting any given host in such an environment, even a less optimal host, is preferable to prolonged exposure to extreme temperatures, desiccation or UV radiation.[15] An alternative hypothesis, substantiated by analyses in our study, could be that these fungi, due to their cosmopolitan distribution, are not host specific. In this case, they would be outcompeted in environments where specialised fungi occur, thereby decreasing their abundance. In harsh environments where lack of plant cover reduces overall propagule number, as well as opportunities to infect plants, the adaptations of these opportunistic colonists for survival in aerosols and in harsh environments might provide them with a competitive advantage. Some of the common taxa isolated in this study are well adapted to live under desert conditions.

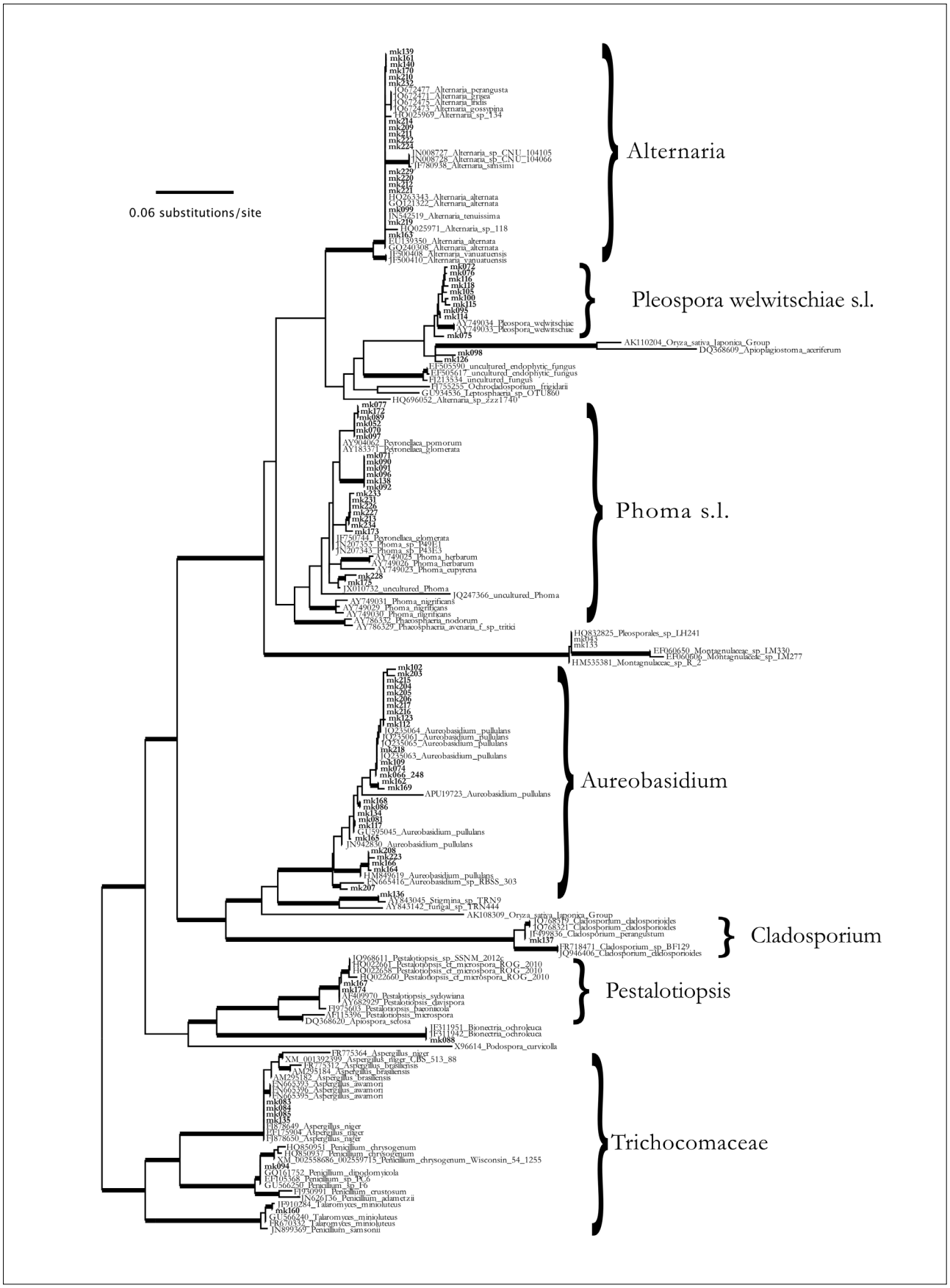

Figure 2: Maximum likelihood reconstruction of Ascomycota groupings isolated from *Welwitschia mirabilis* based on ITS, elongation factor 1 alpha and beta-tubulin 1. A bootstrap of 1000 was conducted. The phylogeny was rooted with members of Eurotiomycetes. Taxa in bold are from this study. Bold branches indicate bootstrap values ≥70. Major fungal lineages are indicated. See also Supplementary data.

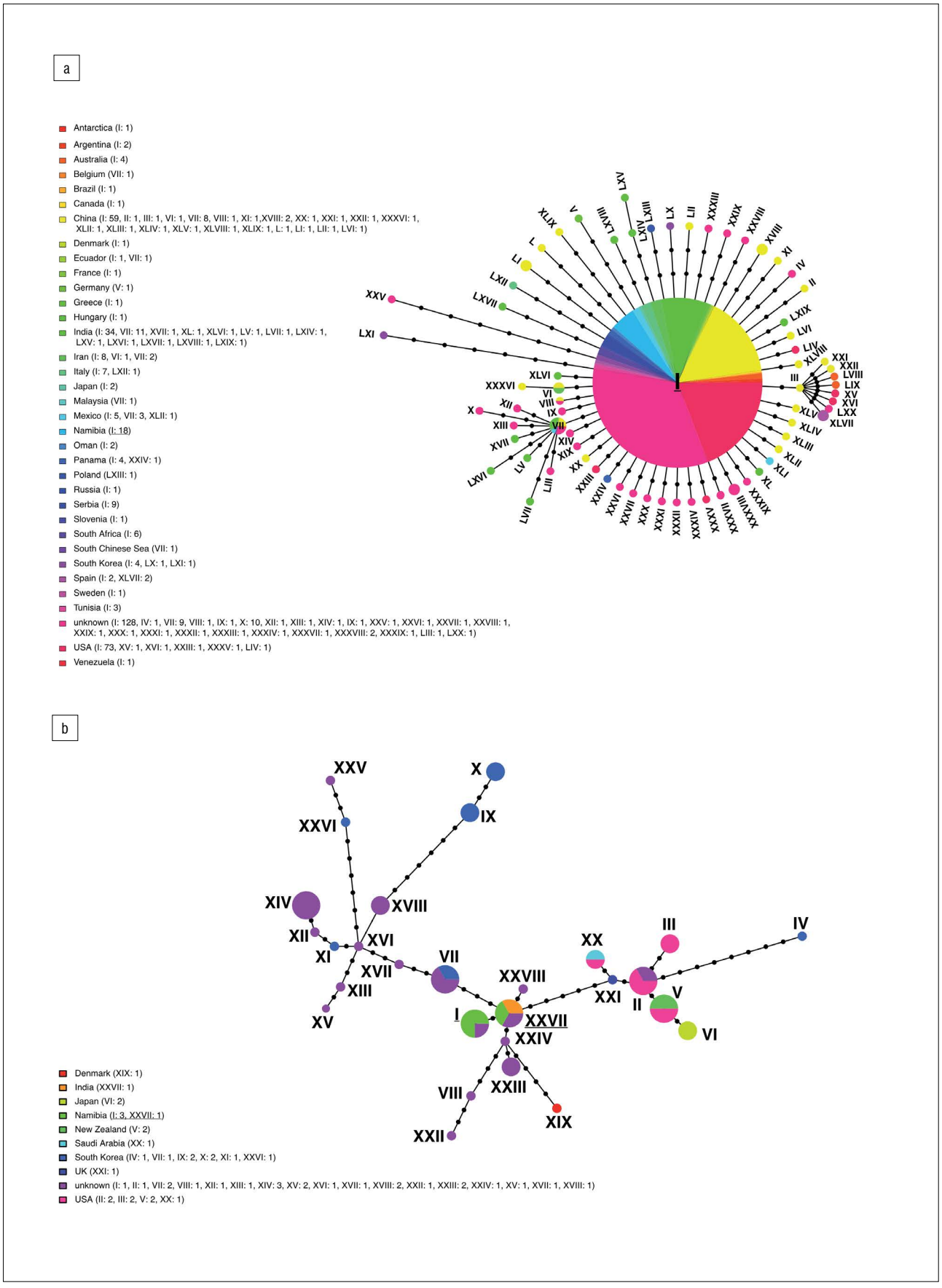

Figure 3: Minimum spanning tree of (a) ITS and (b) beta-tubulin 1 for *Alternaria* sequences. Roman numerals indicate individual haplotypes. Country of origin is colour-coded and text in parentheses indicates the number of sequences per haplotype per country of origin. Haplotypes containing taxa from this study are underlined.

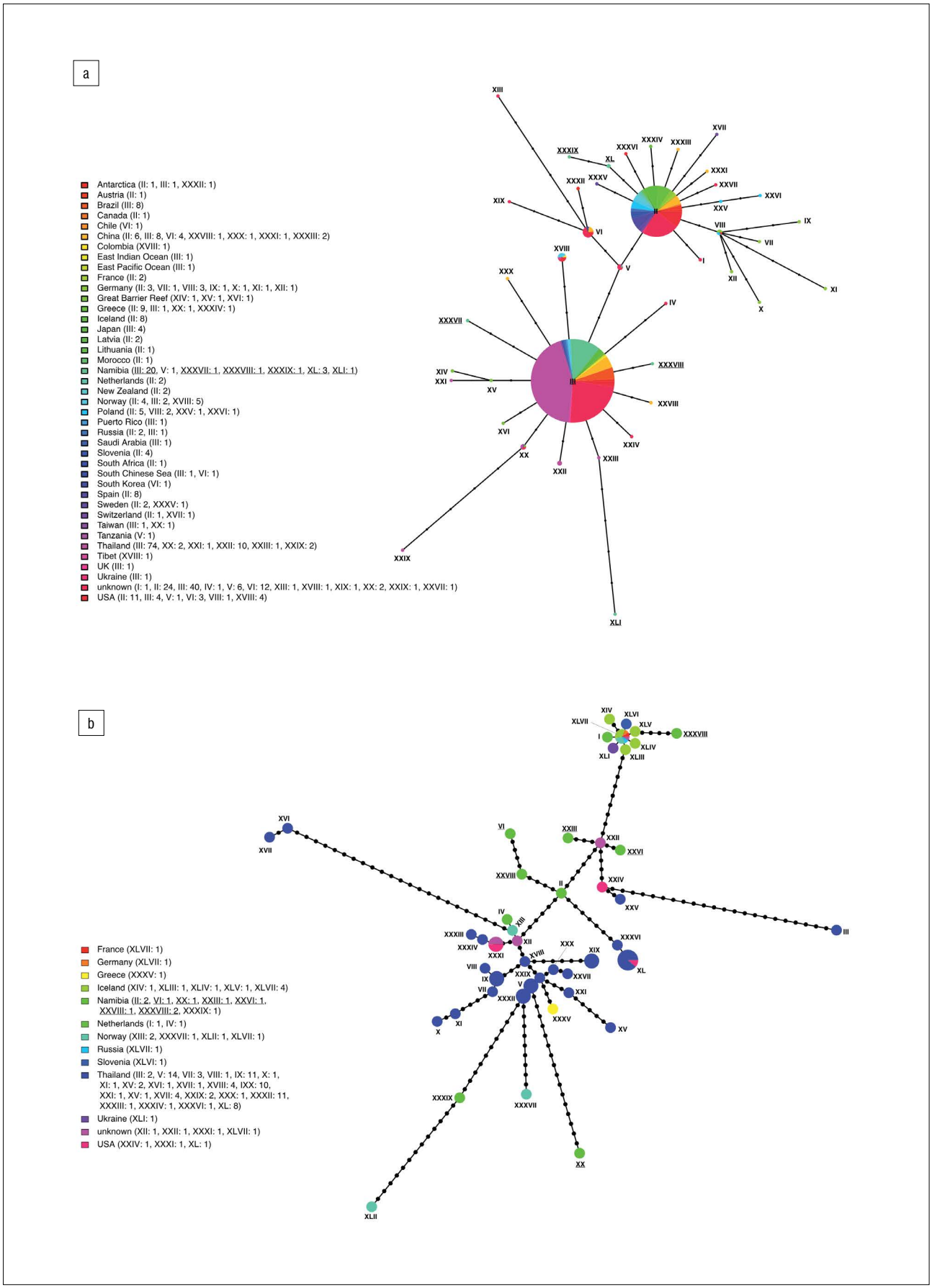

Figure 4: Minimum spanning tree of (a) ITS and (b) beta-tubulin 2 for *Aureobasidium* sequences. Roman numerals indicate individual haplotypes. Country of origin is colour-coded and the text in parentheses indicates the number of sequences per haplotype per country of origin. Haplotypes containing taxa from this study are underlined.

Many of these taxa (e.g. *Alternaria*[58,59], *Aspergillus*[60], *Aureobasidium*[61,62]) have been shown to produce melanins, a group of polymers that fungi produce as protection against harsh environmental conditions.[63] In cases where the fungi enter an endophytic life stage, the production of melanins might be less relevant. However, during long-distance dispersal, arrival, epiphytic growth, and infection in an extreme environment, melanins could be the difference between success and failure of establishment.

In this study, we used a culture-dependent approach to identify fungi. Studies comparing culture-dependent vs. culture-independent next-generation sequencing studies point to the fact that there could be a significant difference in fungi between these approaches.[64] Nevertheless, this culture-based approach remains relevant for the categories of taxa which have been isolated. Future studies, possibly using culture-independent tools and next-generation sequencing, would therefore be especially interesting, particularly with an increased sample size to include gradients from extremely dry to more humid environments. This would make it possible to characterise changes in the patterns common to geographically unique fungal phyllosphere communities. Such studies should be complemented by local air sampling to assess the propagule hypothesis raised in this study.

Acknowledgements

We thank the Department of Science and Technology (DSI)–National Research Foundation (NRF) Centre of Excellence in Tree Health Biotechnology (CTHB) for funding to conduct this study. M.K. thanks the University of Pretoria for postdoctoral funding. We also acknowledge the important improvements suggested by the anonymous reviewers.

Competing interests

We declare that there are no competing interests.

Authors' contributions

D.A.C., B.S. and M.J.W. conceived the study and provided funding. M.K. and D.A.C. conducted the sampling of material. M.K. conducted sample analysis, data analysis and wrote the initial draft. B.S., D.A.C., M.K. and M.J.W. revised the manuscript and wrote the final draft. All authors approved the final draft.

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