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Palm leaf fungi in Portugal: ecological, morphological and phylogenetic approaches

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To my grandpa,
our *little old man*

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Abstract

Palm trees (*Arecaceae*) are a diverse group of monocotyledons distributed across the tropics. Some members of this family are economically important crops and others are highly prized as ornamentals, particularly in Mediterranean climates. Anything that diminishes palm attractiveness, such as foliar lesions, will have a negative impact in their aesthetic value.

In the last 30 years there has been a profusion of studies on palm fungi, a taxonomically diverse and important community. Although most of these studies used a descriptive taxonomy approach, from which many species and genera were formally identified and described as new to science, only some of these studies were focused on biodiversity and/or ecology.

Considering that palm trees are a plant model for studying fungal diversity, and that fungi are one of the main causes of palm foliar lesions, a preliminary assessment of the fungal assemblage on palms was carried out. For this a total of 78 foliar lesions on palm leaves were studied.

Following an isolation flow-chart, a collection of 457 isolates was established. All isolates were characterized according to their macro- and micro-morphology and assigned to genera. Their genetic diversity was assessed by PCR fingerprinting using csM13 and $(GTG)_5$. Diversity indices were computed, and assessments of genera-abundance distribution and genera accumulation curves were also performed. Selected isolates were identified by sequencing of accepted DNA barcodes.

A total of 57 genera were found. The general composition of the fungal community was well described by a log-series model, showing a pattern of dominance of very few genera, and a high profusion of infrequent and rare genera. From the ecological approaches it was found that the fungal communities are subjected to dynamic processes associated with both the host species and the geographical context. Phylogenetic analyses revealed one new genus and four new species, which are here formally described.

This study is pioneer in Portugal, representing a significant advance in the fundamental knowledge on palm fungi.

Keywords: *Arecaceae*, palm fungi, phylloplane fungi, biodiversity indices, ecological approaches, genomic fingerprinting, phylogenetic analyses.

Resumo

As palmeiras são um grupo de plantas monocotiledóneas altamente diversificado pertencentes à família *Arecaceae*. Esta família é representada por cerca de 2600 espécies distribuídas por 181 géneros. As palmeiras são maioritariamente nativas de regiões tropicais e subtropicais, desempenhando um papel importante nos ecossistemas onde se inserem, pela sua elevada adaptabilidade e também pela utilidade dos seus produtos. Entre os membros da família *Arecaceae* encontram-se algumas das culturas de plantas mais cultivadas mundialmente devido à sua importância económica, tais como a tamareira (*Phoenix dactylifera*), o coqueiro (*Cocos nucifera*) e a palmeira-de-dendém (*Elaeis guineensis*). Devido à sua elevada capacidade de adaptação a uma ampla gama de condições climáticas, as palmeiras são muito apreciadas e utilizadas como plantas ornamentais, nomeadamente em climas mediterrâneos. Sendo das plantas mais características dos climas tropicais, as palmeiras conferem às paisagens mediterrâneas o aspecto exuberante e o fascínio dos trópicos, sendo utilizadas com muita frequência em alinhamentos de espaços públicos, em arranjos paisagísticos, na composição de jardins e, ainda, como plantas de interior.

Esta utilização das palmeiras no paisagismo urbano, largamente associado ao desenvolvimento do turismo, fez crescer consideravelmente a utilização destas plantas em países no sul da Europa. A importação das palmeiras das suas regiões nativas, bem como o recente impacto do escaravelho das palmeiras, *Rhynchophorus ferrugineus*, na devastação das palmeiras das Canárias (*Phoenix canariensis*) tem suscitado preocupações relativas a eventuais ameaças fitossanitárias relacionadas com a introdução de agentes fitopatogénicos exóticos, nomeadamente de fungos. Um dos principais tipos de doenças causados por fungos em palmeiras corresponde às lesões foliares. Neste contexto, os fungos associados às lesões foliares de palmeiras constituem o âmbito da presente dissertação.

Em décadas recentes, verificou-se uma grande expressão dos estudos de fungos associados a palmeiras em diferentes áreas da Micologia. Estes fungos começaram a ser considerados uma comunidade taxonomicamente diversa e importante, coloquialmente apelidada de “fungos de palmeiras”. Ainda que a maioria destes estudos tenha utilizado uma abordagem de taxonomia descritiva, a partir da qual inúmeras espécies e géneros foram formalmente identificados e descritos como novos para a ciência, apenas alguns destes estudos tiveram como objecto a exploração da biodiversidade e/ou ecologia dos fungos de palmeiras.

Tendo em conta as características das palmeiras enquanto hospedeiros de excelência para a descoberta de novos fungos, o presente trabalho compreendeu dois objectivos distintos. O primeiro consistiu em avaliar a diversidade e a ecologia dos fungos que se encontram associados a lesões foliares de palmeiras ornamentais em Portugal. O segundo objectivo consistiu em contribuir para a descoberta de novos *taxa* para a ciência, uma vez que esta é actualmente uma das principais tarefas dos micólogos, i.e. o preenchimento da lacuna entre o número de espécies de fungos actualmente descritas e o número de espécies que as mais recentes estimativas preveem. Assim, esta dissertação encontra-se estruturada em duas partes: na Parte I foram aplicadas abordagens ecológicas para o estudo integrado da biodiversidade das comunidades de fungos associadas a lesões foliares de palmeiras e na Parte II foram aplicadas abordagens taxonómicas a determinados isolados, para estudar o seu posicionamento filogenético.

O esforço de amostragem resultou num total de 78 lesões foliares a partir de palmeiras sintomáticas de Oeiras e Lisboa. As lesões foliares foram morfologicamente caracterizadas e serviram como base para o isolamento de fungos a elas associados, para o que foram utilizados vários métodos de isolamento para que as comunidades fúngicas recolhidas se encontrassem devidamente representadas. Em algumas lesões o isolamento compreendeu a observação e manipulação de estruturas fúngicas presentes no tecido hospedeiro. Adicionalmente, todas as lesões foram sujeitas a um método de isolamento directo, o qual compreendeu a

esterilização superficial de porções das lesões, que foram, posteriormente, inoculadas em PDA. Para todos os isolados obtidos foram estabelecidas culturas puras provenientes de um único esporo.

A colecção estabelecida compreendeu 457 fungos filamentosos. Estes foram caracterizados de acordo com as suas características micro- e macromorfológicas, as quais permitiram a sua identificação até ao género. Posteriormente, o DNA genómico foi extraído de culturas de todos esses isolados, utilizando uma versão modificada e optimizada do método do tiocianato de guanidina. Estes extractos foram, seguidamente, utilizados para a obtenção de perfis genómicos de MSP-PCR, os quais foram baseados nos *primers* csM13 e $(GTG)_5$. Os perfis genómicos obtidos foram agrupados em dendrogramas consenso construídos com o *software* BioNumerics, usando o coeficiente de correlação de Pearson e o método de aglomeração UPGMA. A análise destes dendrogramas de perfis genómicos foi utilizada, posteriormente, para avaliar e caracterizar a diversidade genética das comunidades dos fungos isolados. Para além dos métodos clássicos e moleculares de discriminação e monitorização de *taxa* referidos, diferentes análises para a avaliação da diversidade de comunidades de fungos foram aplicadas, incluindo o cálculo de índices de diversidade, a avaliação da distribuição da abundância dos diferentes géneros e, ainda, a avaliação de curvas de acumulação.

Concomitantemente com as análises de biodiversidade e as observações ecológicas, determinados isolados foram seleccionados para a sequenciação de marcadores moleculares actualmente aceites e validados, os quais foram posteriormente utilizados para o seu posicionamento filogenético entre *taxa* actualmente descritos. Para o efeito, foram efectuadas análises filogenéticas por máxima verossimilhança e por máxima parcimónia, com recurso aos *softwares* RAxML e PAUP, respectivamente.

Um total de 57 géneros foi identificado na colecção de isolados estabelecida. A composição geral da comunidade fúngica associada às lesões foliares foi bem descrita por um modelo *log-series*, na medida em que apresentou um padrão bem definido de co-dominância de três géneros, nomeadamente *Alternaria*, *Cladosporium* e *Phoma*, e uma elevada profusão de géneros infrequentes e raros. A considerável diversidade de géneros identificados era expectável e está concomitante com estudos anteriores. Deste modo, verificou-se que a comunidade de fungos associada a lesões foliares de palmeiras apresenta uma ampla distribuição taxonómica, na qual duas ordens do filo *Ascomycota* se encontraram particularmente bem representadas, *Pleosporales* e *Capnodiales*. Apenas um dos isolados obtidos, do género *Graphiola*, pertenceu ao filo *Basidiomycota*, sendo todos os restantes isolados pertencentes ao filo *Ascomycota*, o que também era expectável tendo em conta estudos anteriores.

Na colecção estabelecida, a comunidade de cœlomicetes apresentou uma maior diversidade quando comparada com a comunidade de hifomicetes, na medida em que incluiu a grande maioria dos géneros identificados como infrequentes e raros. Diferenças semelhantes entre cœlomicetes e hifomicetes foram anteriormente reportadas em fungos isolados de palmeiras em climas temperados. Assim, o presente estudo sugere que a diversidade dos fungos de palmeiras em climas temperados parece estar particularmente concentrada na comunidade de cœlomicetes.

As abordagens ecológicas mostraram que as comunidades de fungos isolados estão sujeitas a processos dinâmicos associados quer à espécie do hospedeiro, quer ao contexto geográfico no qual as mesmas foram encontradas. Deste modo, enquanto as palmeiras tipicamente tropicais surgiram, na sua generalidade, empobrecidas em fungos, nas palmeiras tipicamente temperadas as comunidades de fungos foram mais diversas e abundantes, particularmente ao nível dos cœlomicetes. Por sua vez, freguesias nas quais é expectável uma maior humidade relativa, atendendo ao contexto geográfico, apresentaram, também, comunidades de fungos mais diversas e abundantes.

A aplicação integrada dos índices de diversidade, juntamente com a avaliação da distribuição da abundância dos diferentes géneros, previu que pouco mais de 50% da riqueza de géneros foi identificada no

presente estudo. Assim, embora tenha sido registada uma enorme diversidade de géneros de fungos filamentosos, que permitiu em última análise estabelecer tendências ecológicas primárias, o presente estudo está longe de ter registado a potencial diversidade que se encontra associada a lesões foliares de palmeiras em Portugal. Apenas uma abordagem integrada com dados morfológicos e moleculares associada a uma maior amostragem, bem como à execução de réplicas no isolamento, poderá (a) revelar a potencial diversidade destas comunidades de fungos e (b) estabelecer de forma clara as observações e os padrões ecológicos aqui apontados.

As análises filogenéticas revelaram um novo género, *Arecamyces* gen. nov., e quatro novas espécies, *Diaporthe chamaeropsicola*, *Morinia trachycarpae*, *Morinia phoenicicola* e *Arecamyces humilianae* spp. nov., para a ciência, os quais foram formalmente descritos e ilustrados.

O presente estudo, embora baseado numa amostragem relativamente pequena, mostrou que as palmeiras continuam a ser um hospedeiro de excelência para a pesquisa de novos *taxa*, bem como um modelo para estudos de biodiversidade e abordagens ecológicas que possam revelar as dinâmicas das comunidades de fungos. Considerando que apenas 3% dos isolados obtidos foram totalmente caracterizados, espera-se que outros novos *taxa* sejam descritos em estudos futuros. Por fim, este estudo de fungos associados a lesões foliares de palmeiras ornamentais em Portugal é pioneiro e representa uma inovação na investigação do conhecimento fundamental.

Palavras-chave: *Arecaceae*, fungos de palmeiras, fungos do filoplano, índices de biodiversidade, abordagens ecológicas, perfis genómicos, análises filogenéticas.

Presentations and future papers based on this dissertation

Parts of this dissertation have been submitted as an abstract for poster presentation at Microbiotec'19, Coimbra, Portugal, 5th–7th December 2019:

> Pereira DRS, Tenreiro R and Phillips AJL. 2019. First survey of microfungi from palm leaf spots in Portugal reveal a remarkable mycobiota diversity. Poster presentation in Microbiotec'19, Coimbra, Portugal.

Case Study I will be published, with modifications, in Fungal Diversity Notes 13, Fungal Diversity (2020).

Case Study II will be published in Mycosphere (2020).

Case Study III will be published, with modifications, in Phytopathologia Mediterranea (2020).

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List of Abreviations

BLAST	Basic Local Alignment Search Tool
CI	Consistency Index
CIPRES	Cyberinfrastructure for Phylogenetic Research
DIC	Differential Interference Contrast
GTR	General Time Reversible
HI	Homoplasy Index
ITS	Internal Transcribed Spacer
LSU	Large SubUnit
ML	Maximum Likelihood
MP	Maximum Parsimony
MSP-PCR	Microsatellite/Minisatellite Primed-PCR
NCBI	National Center for Biotechnology Information
PAUP	Phylogenetic Analysis Using Parsimony
RAxML-HPC2	Randomized Axelerated Maximum Likelihood for High Performance Computing
RC	Rescaled Consistency index
RI	Retention Index
TBR	Tree Bisection and Reconnection
TL	Tree Length
UPGMA	Unweighted Pair Group Method with arithmetic Average

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GENERAL INTRODUCTION

Palm trees as a plant model for studying fungal diversity: historical overview and actual biodiversity challenges

1. Palm trees, a distinctive element in the urban landscape
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1. Palm trees, a distinctive element in the urban landscape

Palms are perennial monocotyledonous trees in the family *Arecaceae* (syn. *Palmae*), and one of the most distinctive of all plants families since they are easily recognized from their morphology. These trees are almost exclusively tropical or sub-tropical, insomuch as they are considered to be "emblems of the tropical forests" where their natural biodiversity is concentrated (Tomlinson, 2006; Dransfield *et al.*, 2008; Eiserhardt *et al.*, 2011; Balslev *et al.*, 2016). Very few species are native of temperate climates (Eiserhardt *et al.*, 2011). Geographically palm trees can be found in habitats ranging from southern France, where the European fan palm *Chamaerops humilis* is native (44° north latitude), to New Zealand, where the shaving brush palm *Rhopalostylis sapida* naturally occurs (44° south latitude) (Johnson, 2011). This remarkable extent of latitude where palms can grow reflects their impressive adaptability to diverse climatic conditions, which can be seen by the abundance of these trees in temperate regions, although the overwhelming majority of palm species are native to tropical climates, particularly to the Malaysian and Neotropical regions. The ability to grow under different climatic conditions reflects the enormous diversity of habitats, from tropical rainforests to deserts, that are exploited by palm trees (Walther *et al.*, 2007; Eiserhardt *et al.*, 2011; Brancalion *et al.*, 2018).

Ecologically, palm trees are typically conspicuous large-bodied plants that often have a major ecological impact in the plant communities present in the different habitats occupied, especially in tropical ecosystems (Couvreur and Baker, 2013). They provide important food resources not only to the fauna, but also to the indigenous communities of these regions, who use the trees for their daily needs. These comprise an extensive range of traditional uses, from thatching, basketry, weaving and house construction to multiple medicinal purposes (Baker *et al*., 2009; Johnson, 2011). Indeed, palms are among the best known and most extensively cultivated plant families and have been recognized, along with the *Poaceae* and the *Fabaceae*, as being one of the three most important and useful plant families in terms of human exploitation (Tregear *et al.*, 2011; Johnson, 2011). Besides the usage in their native, mainly tropical, range, these plants are also important in terms of worldwide usage. Almost all parts of palm trees can have an economic value with many uses in different species, assuming an extremely economic importance in the international trade where oil (*Elaeis* species), coconut (*Cocos nucifera*), rattan (*Calamus* species) and date (*Phoenix dactylifera*) palms are particularly relevant (Tomlinson, 1979; Baker *et al*., 2009; Johnson, 2011).

According to the most recent estimates, *Arecaceae* family, the only in the order *Arecales*, comprises around 2600 species in 181 genera, among which are included some of the most graceful, attractive and majestic forms of the *Plantae* kingdom with an enormous diversity of features and aspects (Dransfield *et al.*, 2005, 2008; Asmussen *et al*., 2006; Trias-Blasi *et al.*, 2015; Baker and Dransfield, 2016). This renders them the category of interesting objects in natural and artificial landscapes. Thus, besides the economic relevance of the previously cited important crops, palm trees are also highly prized and used as ornamental plants in the most varied places. They are extensively used in parking lots, along city streets and boulevards, in shopping centers, on hotel grounds, in public and private gardens, near swimming pools and in orchards, botanical gardens and parks, since they offer a great decorative potential in any landscape (Ramos *et al.*, 2013, 2015; Beaudoin-Ollivier *et* al., 2017; MacLeod and Hussein, 2017). There are many kinds of palms with a wide range of growth habits appropriate for any environment and landscape site, besides the fact that these trees are durable, long-lived and easy to care for (Riffles *et al.*, 2003; Broschat *et al.*, 2014; Beaudoin-Ollivier *et* al., 2017; Cohen, 2017). In Portugal, palms are grown solely as ornamentals for their attractiveness as decorative plants in parks, gardens and cityscapes. One single palm species, *Chamaerops humilis*, is native to Portugal, with a natural occurrence in the Algarve region (Fabião and Oliveira, 2006; CML, 2015; ICNF, 2016). All the other palms planted in portuguese cities, particularly *Phoenix dactylifera*, *Washingtonia filifera*, *Washingtonia robusta*, *Trachycarpus fortunei* and, especially, *Phoenix canariensis* (Ramos *et al.*, 2013, 2015; CML, 2015), are exotic. Besides the usage of these trees in landscaping arrangements and composition of gardens, they are also used as indoor plants, with *Dypsis lutescens* and *Chamaedorea elegans* the most used in Portugal.

Around the Mediterranean, palm trees have become an essential and distinctive component of the urban landscape, where they provide a bold and luxuriant look typical of the tropical flora, which sometimes is used

as a symbol of tourism (Ramos *et al*., 2015; MacLeod and Hussein, 2017). Consequently, the use and importation of these plants have expressively increased over the last decades, which has raised new phytosanitary threats, such as the red palm weevil *Rhychophorus ferrugineus*, and highlighted the potentially devastating effects of pests and diseases introduced onto a host growing outside of its native range (Ramos *et al.*, 2013, 2015; Beaudoin-Ollivier *et* al., 2017; MacLeod and Hussein, 2017). In fact, the rapid increase in the international trade of plants, most of which are used as ornamentals, is a major pathway for the spread of associated pests and other invasive alien species (Parker and Gilbert, 2004; Desprez-Loustau *et al*., 2010; Gilbert and Parker, 2010; Lilja *et al*., 2011; MacLeod and Hussein, 2017). Like any other plant, palm trees are prone to attack by pathogens, especially fungal pathogens, and subject to diseases and pests that can be responsible for considerable damage, whether in outdoors or indoors plants. Since these plants are an exotic element in Portugal's urban landscapes, the potential introduction of alien fungal pathogens is a threat whose study should be of utmost status. Considering that palms in Portugal are grown exclusively for the beauty of their foliage, leafspots and leaf blights, although responsible for relatively minor damage, play an important role in palm trees diseases because they disfigure the foliage, which consequently depreciates their aesthetic value. Since fungi are the main cause of these diseases (Elliott, 2018a) it is important to have knowledge on the fungi associated with them, which is the scope of this dissertation.

2. Palm fungi, an historical overview

The study of microfungi associated with palm trees makes it possible to explore different types of research in the Mycology area. While the majority of previous studies on palm fungi have focused on descriptive taxonomy (Hyde, 1992a; Pinnoi *et al.*, 2003a; Konta *et al.*, 2016c), a considerable number of studies explored the biodiversity and/or the ecology of these microorganisms (Yanna *et al.*, 2002; Pinnoi *at al*., 2006; Sarma and Hyde, 2018; Monteiro *et al*., 2019). The following approach intends to give a general and brief overview of the research that has been done on this subject. It should be noted that the relevance of the studies on microfungi associated with palms made them begin to be considered an important and taxonomically diverse assemblage that is often referred to as "palm fungi" or "palmicolous fungi" (Taylor *at al*., 2000; Hidayat *et al*., 2006). This terminology is adopted herein.

Systematic and descriptive taxonomy studies

There are numerous reports scattered through the literature of fungi collected from different parts of different species of palm trees and from different regions of the world. An overwhelming number of these studies was dedicated to collection and description of fungi, including new genera and new species. Although there are several reports prior to the 1990s, the present overview focuses on the extensive studies carried out by Hyde and his co-workers. Most of the descriptions before Hyde's research comprise short Latin paragraphs that lack illustrations and give limited information about the true identity of the fungi (Hyde, 1993h). Nevertheless, it is noteworthy to mention two extensive studies on palm fungi, namely those of Pirozynski and Matsushima. Pirozynski (1972) reported 46 species, including one new genus, *Bondiella*, six new species and some new combinations, of fungi from oil palm *Elaeis guineensis* collected from Tanzania. On the other hand, Matsushima (1971, 1975, 1980, 1981, 1983, 1985, 1987, 1989, 1993 and 1995) reported 326 fungi from palm litter, including one new genus, *Venustocephala*, and 88 new species, collected from different regions around the globe, from the Americas to Australia and Eastern Asia, most of which were compiled in the classic book series "Matsushima mycological memoirs". In the last 30 years, mycology has been observing an extensive profusion of studies regarding palmicolous fungi by Hyde and his co-workers. These systematic studies culminated in the publication of three books entitled "Palm microfungi" (Fröhlich and Hyde, 2000), "Genera of ascomycetes from palms" (Hyde *et al*., 2000) and "Microfungi of tropical and temperate palms" (Taylor and Hyde, 2003), and a series of publications entitled "Fungi from palms", comprising 40 papers where a lot of new fungi to science were described (Hyde, 1992b,c, 1993a–h, 1994a–d, 1995a–g, 1996a–g; Fröhlich and Hyde, 1995a, 1998a,b; Hyde and Fröhlich, 1995a, 1997; Hyde and Taylor, 1996; Hyde *et al*., 1996a, 1998a,b, 1999a, 2000; Hyde and Aptroot, 1997; Taylor *et al.*, 1997; Lu and Hyde, 1999; Taylor and Hyde, 1999a; Aptroot *et al.*, 2000; Fröhlich *et al.*, 2000b; Guo and Hyde, 2001; Hosagoudar

et al., 2001; Sarma and Hyde, 2001; Smith and Hyde, 2001). Genera of fungi with common representatives found on palms are summarized in Supplementary Table A.1. Genera that were described as new to science and found on palms in the last three decades are summarized in Supplementary Table A.2.

Microfungi from palms have been studied since 1988 and 1989 with the description of the genera *Linocarpon* and *Oxydothis* from the mangrove palm *Nypa fruticans* (Hyde, 1988; Hyde and Nakagiri, 1989). Hyde made an extensive survey of these palmicolous fungi from a wide range of tropical palm species in different regions of the world, such as Brunei (Hyde, 1991a,b, 1992d, 1993f, 1994a, 1996d,g), Indonesia (Hyde, 1993f,g, 1995c,e, 1996d,e,f, 1997b), Ecuador (Hyde, 1995g, 1996g, 1997a), Papua New Guinea (Hyde, 1994e,f, 1995f, 1996f), Malaysia (Hyde, 1993c, 1994b,c, 1996d,f, 1997b), Australia (Hyde, 1993a,b,e,h, 1996e,g, 1997b), Philippines (Hyde, 1995d) and USA (Hyde, 1995a,c). All the regions surveyed revealed the presence of an enormous diversity of fungi, among which many genera were described as new to science (Supplementary Table A.2). Along with Hyde's work on redescribing, illustrating and monographing existing genera (Hyde, 1992b, 1993a,d, 1994c, 1995e,f, 1996a,c), these studies resolved certain taxonomic relationships. A striking example includes the description of numerous new species from the genera *Linocarpon* (Hyde, 1988, 1992a; Hyde and Alias, 1999) and *Oxydothis* (Hyde and Nakagiri, 1989; Hyde, 1993d, 1994c), two of the most common genera found on palms (Supplementary Table A.1). These first case studies in the early 90s verified the existence of a well-represented set of morphological characters, which allowed to distinguish these genera from other related genera, as well as to clarify the existence of new morphologically similar genera.

The huge number of new genera and new species reported on palms by Hyde in the early 90s, rapidly increased the interest in the mycobiota of this host and several of his students and co-workers worked towards extending the knowledge on palmicolous fungi. Thus, different aspects of these fungi were studied and contributed on the way to the fundamental fungal and biodiversity knowledge, besides the many new taxa reported. The intensive research led, ultimately, to description of at least two knew families, namely *Phaeochoraceae* (Hyde *et al.*, 1997a) and *Apiosporaceae* (Hyde *et al.*, 1998b) to accommodate genera that were described on palms, respectively, *Cocoicola* (Hyde, 1995b) and *Appendicospora* (Hyde, 1995d), along with extant related genera. In fact, the constant description and illustration of new taxa found on palms lead frequently to the discussion of their placement in fungal classification (Yanna *et al.*, 1997; Hyde *et al.*, 1998b, 2000; Taylor *et al.*, 2001).

Hyde, Fröhlich, Taylor, Aptroot and Goh, studying ascomycetes developing on living, diseased and dead palm material, surveyed different regions from East and Southeast Asia (Hyde and Fröhlich, 1995a, 1997; Goh and Hyde, 1997b; Hyde *et al.*, 1997b, 1998a, 1999a, 2000; Taylor *et al.*, 1997, 1999; Taylor and Hyde, 1999a; Aptroot *et al.*, 2000), Australasia (Taylor *et al.*, 1996; Goh and Hyde, 1997a; Hyde and Fröhlich, 1997; Fröhlich and Hyde, 1998b; Hyde *et al.*, 1998a, 1999a,d, 2000; Fröhlich *et al.*, 2000b) and South America (Fröhlich and Hyde, 1995a; Goh and Hyde, 1996a,b, 1997b; Hyde and Fröhlich, 1997; Taylor and Hyde, 1999b) and reported more than 50 new species of ascomycetes. One intensively studied region worth mentioning is the rainforests of North Queensland (Australia), where Fröhlich and Hyde (1994, 1995b,c,d, 1998b), Hyde and Fröhlich (1995b) and Fröhlich *et al.* (1997, 2000b) found an immense diversity of fungi, some of which were associated with palm leaf spots. These studies yielded, for example, the new genus *Maculatipalma* (Supplementary Table A.2). Besides the incredible contribution towards the knowledge on fungal biodiversity and its placement in fungal classification, these studies also allowed to discuss and unveil some sexual-asexual morphs connections (Hyde *et al.*, 1996b; Jones *et al.*, 1996, Hyde *et al.*, 1999d).

The studies on palmicolous fungi in the tropics proceeded with other regions starting to be surveyed more systematically, including many reports from additional Hyde co-workers, namely Yanna, Pinnoi, McKenzie and Pinruan. Yanna *et al.* (1997, 1998a,b, 1999, 2000b, 2004) described several new species from different palm tree species in Hong Kong, comprising the typical *Ascomycota* assemblage commonly found on palms, particularly with species of *Appendicospora* (Yanna *et al.*, 1997), to atypical hyphomycete and coelomycete genera, such as *Koorchaloma* (Yanna *et al.*, 1998a), *Staurophoma* (Yanna *et al.*, 1998b) and *Endomelanconium* (Yanna *et al.*, 1999). Other hyphomycetes were described from palms during similar studies in Brunei, Thailand and North Queensland (Yanna *et al.*, 2000a, 2001c; Hyde *et al.*, 2002). Although

the initial studies on palms focused mainly on the evaluation of *Ascomycota* coverage through the presence of its sexual morphs on the host, particularly on mangrove palms such as *Nypa fruticans*, the evident potential of these hosts for biodiversity surveys led to the diversification of both the approaches and, consequently, the discoveries. Thus, a considerable number of reports started to describe several new species of palmicolous hyphomycetes, in addition to the usual ascomycetes sexual morphs, from already well surveyed regions, such as Brunei, Hong Kong, North Queensland and Thailand (Goh and Hyde, 1997a,c, 1998a,b,c, 2000; Goh *et al*., 1999; Hyde *et al.*, 1999b; Ho *et al.*, 2001; Wong *et al*., 2001, Yanna and Hyde, 2002; McKenzie *et al.*, 2002; Pinruan *et al*., 2002, 2004a,b; Pinnoi *et al.*, 2003a,b, 2004) (Supplementary Table A.1). Along with these new palmicolous hyphomycete species, four new genera were described as new to science, namely *Stratiphoromyces* (Goh and Hyde, 1998b), *Polybulbophiale* (Goh and Hyde, 1998c), *Mackenziella* (as *Mackenziea*) and *Waihonghopes* (Yanna and Hyde, 2002) (Supplementary Table A.2), the last two resulting from the extensive investigation on the biodiversity of fungi on palms in North Queensland previously mentioned.

A perusal of the available literature on palm fungi reveals that no intensive studies on palmicolous hyphomycetes and, especially, coelomycetes have been carried out so far. Nevertheless, along with the previously cited examples from Hyde and co-workers, some scattered studies reporting new species and/or genera of palmicolous hyphomycetes are worth mentioning. Subramanian (1992, 1993, 1994, 1995, 1996) reported several new species and four new genera (Supplementary Table A.2) of dematiaceous hyphomycetes from different palm species in Malaysia and India. Sierra *et al*. (1997a,b,c, 1998) and Abarca *et al*. (1997) described several new hyphomycetes from palms in Central American countries, including Costa Rica, Mexico and Cuba. Later Castañeda-Ruíz *et al*. (2000, 2001) surveyed palmicolous fungi from litter of forests in the Canary Islands and Venezuela and Delgado (2008a,b, 2009, 2010, 2013, 2014) reported several new species of palmicolous hyphomycetes on dead plant debris from South Florida, including two new genera, namely *Veramycella* (Delgado, 2009) and *Kalamarospora* (Delgado, 2010) (Supplementary Table A.2).

A few scattered studies have surveyed palmicolous fungi in Argentina. However, they were not systematic studies concerning descriptive taxonomy, but studies done to understand better the diversity of ascomycetes on woody parts of palms, especially in areas or parks that had been proposed as natural reserves for protection (Capdet and Romero, 2010, 2012; Capdet *et al*., 2010; Trierveiler-Pereira *et al*., 2012). In a similar way, considering the available literature on palm fungi, except for some stray collections, no intensive studies on the fungal diversity on palms in India have been carried out. Nevertheless, a few reports reveal a remarkable diversity of palmicolous fungi, namely those of Subramanian (1952a,b, 1953, 1955) that, in his series "Fungi imperfecti from Madras", reported new hyphomycetes species from dead palm leaves in Chennai, besides his previous cited reports of dematiaceous hyphomycetes (Subramanian, 1992, 1993, 1994, 1995, 1996), and D'Souza and Bhat (2002) and D'Souza *et al*. (2002), who reported several new species and one new genus (*Ernakulamia*) of dematiaceous hyphomycetes (Supplementary Table A.2) from forests in India.

This overview of the literature shows that palms support a vast array of fungi, especially ascomycetes. In fact, in the well surveyed tropical regions of East and Southeast Asia, Australasia and South America, where these fungi have received considerable attention, a remarkable diversity of fungi was revealed with the description of numerous new species. Although much of this diversity could be attributed to the tropical and subtropical habitats surveyed, the few research works done on palms that can thrive in temperate regions, such as New Zealand (Taylor and Hyde, 2003; Mckenzie *et al*., 2004) and some countries of Europe (Taylor and Hyde, 2003), also showed a considerably rich diversity of fungal assemblages, where some new taxa were discovered as new to science. Additionally, as can be verified from the previous brief literature review, the diversity of palmicolous fungi recovered can in part be due to the wide range of hosts and habitats surveyed, including different palm species in terrestrial, freshwater and marine or mangrove ecosystems.

Up to 2003, the intensive research carried out by Hyde and his co-workers discovered and reported, with full descriptions and illustrations, more than 280 species of palmicolous fungi belonging to more than 150 genera (Taylor and Hyde, 2003). Nevertheless, in all these earlier studies, all taxa reported were introduced, described and arranged in different taxonomic ranks within the *Ascomycota* based solely on their morphology (Fröhlich and Hyde, 2000; Hyde *et al*., 2000; Taylor and Hyde, 2003). This approach was, however,

subjective and many taxa were assigned to *Ascomycota* genera *incertae sedis*. Therefore, considering that palms are important hosts harbouring potential novel fungal species, it is critical that these palmicolous fungi are recollected, epitypified where needed, isolated and sequence data obtained so that they can be placed in a natural taxonomic framework (Ariyawansa *et al*., 2013, 2014; Jayasiri *et al*., 2015). Referring to the initial striking example concerning some of the most common fungal genera found on palm trees, such as *Linocarpon*, *Oxydothis* and *Neolinocarpon*, several species were report from palms, but only a small percentage of them have associated sequence data available on accessible databases, what makes their position in the natural taxonomic framework weakly supported as pointed out by Konta *et al*. (2017). Thus, the known taxa, introduced based solely on morphological characteristics, need to be recollected so that molecular data can be used to establish their natural phylogenetic placements.

Relatively few of the earlier studies on palmicolous fungi were based on molecular data. However, the introduction of molecular methods for the study of fungi served as a stimulus for the description of new taxa from palms. Some of the first researches on palmicolous fungi that combined molecular with morphological data were those from Pinruan *et al*. (2004c,d, 2008, 2010) and Pinnoi *et al.* (2010) in Thailand, who reported several new taxa, including new genera (Supplementary Table A.2), of saprobic fungi on the peat swamp palms *Licuala longecalycata* and *Eleiodoxa conferta*. This constitutes a continuation from the previous solely morphological studies that already yielded a remarkably rich diversity of fungal taxa (Pinruan *et al.*, 2002, 2004a,b; Pinnoi *et al.*, 2003a,b, 2004; Liu *et al*., 2011a). The number of reports increased rapidly revealing not only several new taxa, but also resolving their phylogenetic position among closely related taxa. This, in turn, contributed towards unveiling the natural taxonomic framework of the palmicolous fungi, including members of the classes *Sordariomycetes* (Bahl *et al*., 2005; Hidayat *et al.*, 2006; Pinruan *et al*., 2008, 2010; Pinnoi *et al.*, 2010; Geng *et al*., 2013; Daranagama *et al*., 2016; Konta *et al*., 2016b; Delgado and Miller, 2017) and *Dothideomycetes* (Pinnoi *et al.*, 2007, 2010; Phillips *et al*., 2008; Liu *et al*., 2010, 2011b; Wulandari *et al*., 2011; Konta *et al*., 2016a,c; Delgado *et al.*, 2018; Zhang *et al.*, 2018) frequently found on palms. The latest reports on palmicolous fungi have included the establishment of new families to accommodate extant and new palmicolous taxa, including the new genera *Longicorpus* and *Striatiguttula* from *Nypa fruticans* in Thailand (Konta *et al.*, 2017; Li *et al.*, 2017; Zhang *et al.*, 2018, 2019) (Supplementary Table A.2). Thus, these studies continuously reveal not only the importance of the *Arecaceae* family as a host for the search for new fungal species, but also the imperative need of applying sequence data to resolve the phylogeny of this taxonomically diverse assemblage of palmicolous fungi.

Biodiversity and ecological studies

As stated before, palm trees have shown to be a diverse habitat exhibiting an intense fungal colonization. The great majority of studies on microfungi colonizing palms are taxonomic, insomuch as they have focused primarily on cataloguing fungi and describing new taxa collected from several regions around the globe, especially in the tropics. Nevertheless, a few studies concerning palmicolous fungi are ecological and have concentrated on the biodiversity and ecology of saprobic and endophytic fungi. Although the approach of these studies is different, the description of new taxa often resulted from initially ecological approaches that yielded several interesting fungi to properly analyze. This suggests that both approaches may be crucial to explore the fungal knowledge base and its biodiversity.

Briefly reviewing the literature on ecology of palm fungi reveals that studies have been done over the last three decades. Fröhlich and Hyde (1999) studied the biodiversity of palm fungi in the tropics, raising the question that the estimate of 1.5 million would be a "very conservative estimate of the number of fungal species extant on the planet". Taylor *et al*. (2000) investigated the biogeographical distribution of microfungi from temperate and tropical palms, revealing that differences in fungal assemblages were more related to climatic influences than hosts sampled. In addition, Yanna *et al*. (2001a,b, 2002) following her previous cited studies on palmicolous fungi from Hong Kong, assessed the composition of fungal communities and its succession over time, pointing out that differences in fungal assemblages could be related to different growth stages, different sites, different hosts and different tissues sampled. Besides these, several reports were dedicated to endophytic palmicolous fungi, one of the first ecological issues to be investigated on fungi from

palms (Rodrigues and Samuels, 1990; Rodrigues *et al.*, 1993; Rodrigues, 1994; Southcott and Johnson, 1997; Guo *et al.*, 1998; Fröhlich and Hyde, 1999; Fröhlich *et al.*, 2000a). Such studies frequently reported significant differences in the number of isolates in respect to plant growth stages, season, site and tissues sampled, not only on tropical palms within their natural geographic range, but also on temperate palms, such as *Trachycarpus fortunei* (Taylor *et al.*, 1999). The importance of the subject and its implications in fungal biology rapidly increased the interest of mycologists on unveiling several aspects of the ecology of palmicolous endophytes. Molecular data then started to be applied in such studies and made endophytes one of the main subjects of palmicolous fungi to be explored until recent years (Guo *et al.*, 2001; Gómez-Vidal *et al.*, 2006; Rungjindamai *et al.*, 2008; Pinruan *et al.*, 2010; Jiaojiao *et al.*, 2015, 2016; Mahmouda *et al.*, 2017). Relatively few studies have focused on palmicolous pathogens (Fröhlich *et al.*, 1997; Hyde and Cannon, 1999). Other ecological studies have focused on fungi from peat swamp palms (Pinnoi *et al.*, 2006; Pinruan *et al.*, 2007) and mangrove palms (Hyde and Alias, 2000; Pilantanapak *et al.*, 2005; Hyde and Sarma, 2006; Loilong *et al*. 2012; Sarma and Hyde, 2018). One of the most recent publications in the ecology of palmicolous fungi reported aspects related to the existence of fungal species on *Nypa fruticans* at Brunei, which unveiled some aspects related to the fungal community structures and helped in understanding ecosystem dynamics (Sarma and Hyde, 2018).

The ecological studies are extremely important, along with the taxonomic approaches, to assess a complete and integrated perspective of the biodiversity surveys, since biodiversity relies both on the taxa and on their biotic and abiotic interactions (Zak and Willig, 2004). In fact, the high fungal diversity that has been reported on palm trees could be associated with specific ecological questions, such as any kind of site, host or, more intrinsically, tissue specificity of the fungal assemblages surveyed, or even any kind of biotic relationships established between them.

3. Palm fungi, an assemblage with a key role in biodiversity surveys

Regular discoveries of new fungal species have incited mycologists to wonder about the number of fungi that exist worldwide. Since Fries (1825), who established a comparison between fungi and insects diversity, fungi are known as one of the largest groups of organisms. Estimates of the number of fungal species worldwide has varied over time, comprising from relatively low numbers of 100 000 (Bisby and Ainsworth, 1943), 270 000 (Martin, 1951) and 720 256 (Schmit and Mueller, 2007) to impressive higher estimates of 3.5 to 5.1 (O'Brien *et al*., 2005) and almost 10 million (Cannon, 1997). Until recently, the most cited and acknowledged number was the 1.5 million fungal species hypothesized by Hawksworth (1991), who based his conclusions on the observed ratio between flowering plant diversity and fungal diversity. However, this number was considered "a very conservative estimate" and has been revisited several times in the literature as the world rate of description of new species has increased over the last decades (Fröhlich and Hyde, 1999; Hawksworth, 2001, 2004, 2012; Bass and Richards, 2011; Blackwell, 2011).

The currently accepted estimate of species richness is between 2.2 to 3.8 million (Hawksworth and Lücking, 2017). Considering that presently 120 000 accepted fungal species are known, these values indicate that less than 10% of the worldwide mycota is named so far. For that reason, the question "where are the missing fungi?" has often been asked (Hawksworth and Rossman, 1997; Fröhlich and Hyde, 1999; Hyde, 2001; Tang *et al*., 2006, Hyde *et al*., 2007; Mueller and Schmit, 2007). This in turn has motivated the persistent search for new fungal species. To search for these undescribed fungal species is important for biodiversity surveys, which have an incredible economical potential in discovering organisms with novel biotechnological and industrial uses (Hyde *et al*., 2019b). Palm trees have proven to be a rich source of new fungal taxa, and a remarkable number of new species to science have been described from this host over the last three decades (Fröhlich and Hyde, 2000; Hyde *et al*., 2000; Taylor and Hyde, 2003; Zhang *et al*., 2019). The inventory of fungal species from different substrata, particularly niches where species richness is very high, such as palm trees, certainly account for the description of some of the missing fungal diversity (Hyde, 2001; Hyde *et al*., 2007). Indeed, the evidence gained from the extensive palm fungi research undoubtedly indicates that many of the missing fungi can be found on palms, insomuch that Hyde *et al*. (1997c) discovered that 75% of the fungi collected from palms were new to science.

Palm fungi are a taxonomically diverse group, comprising more than 1500 described species, which include representatives from almost all major fungal classes (Fröhlich and Hyde, 2000; Hyde *et al*., 2000; Taylor and Hyde, 2003; Zhang *et al.*, 2019). The most representative group of palmicolous fungi are the ascomycetes, a diverse assemblage within which the best represented family is *Xylariaceae* (Zhang *et al*., 2019). Concerning higher taxa, the *Sordariomycetes* are the best represented class, with three commonly recorded genera, namely *Anthostomella*, *Linocarpon* and *Oxydothis* (Taylor and Hyde, 2003; Hidayat *et al*., 2006, 2007; Lechat and Fournier, 2012; Daranagama *et al*., 2016; Konta *et al*., 2016b, 2017). Recent reports, particularly those based in both morphological and phylogenetic data, have described a series of *Dothideomycetes* within which the best represented fungal genera are members of the orders *Pleosporales*, such as astrosphaeriella-like species, *Fissuroma* and *Roussoella*, and *Botryosphaeriales*, such as *Botryosphaeria*, *Phyllosticta* and *Neodeightonia* (Liu *et al*., 2010, 2011b, 2012, 2014; Wulandari *et al*., 2011; Phookamsak *et al.*, 2015; Wanasinghe *et al*., 2018; Zhang *et al.*, 2018; Nuankaew *et al.*, 2019).

The great diversity of fungi that has been recorded on palms plays an important role in different aspects related to biodiversity surveys.

Firstly, the studies by Hyde and his co-workers have resulted in a wealth of data that provided new information for fungal biodiversity estimates and established a much higher fungus to plant ratio ranging from 26:1 to 33:1 (Hyde, 1995h; Fröhlich and Hyde, 1999; Hyde *et al*., 1997c). These estimates imply the existence of almost 73 thousand species of fungi on palms worldwide of which only less than 3% are currently known (Taylor and Hyde, 2003). In this wealth of data, a diverse and abundant assemblage of host- (Hyde and Alias, 2000) and tissue-specific fungi (Yanna *et al*., 2001b; Pilantanapak *et al*., 2005; Pinnoi *et al*., 2006) was found, which account for the high fungal diversity reported on palms, subsequently accounting for the largely unknown species estimated.

Secondly, studies on palmicolous fungi led to discussions about the effect of geographical location on composition of the mycobiota, since different composition was significantly affected on same host taxa occurring in different countries, which have important implications in fungal estimates and ecology (Taylor *et al*., 1999; Yanna *et al*., 2001a). Considering that the great majority of palms are native of tropical climates, the study of their fungal assemblage also addressed the issue concerning the global fungal diversity differences between temperate and tropical climates (Fröhlich and Hyde, 1999; Taylor *et al*., 2000).

Thirdly, palms trees have been revealing interesting aspects of endophytes, not only because they comprise a considerable amount of them, as assessed by Guo *et al*. (1998) studies on palm endophytic sterile mycelium, but also because they allowed observations on the changing of endophytes mode of life to saprophytes once the host tissue died (Fröhlich and Hyde, 2000; Guo *et al*., 2001).

Finally, the evidence gained from the extensive palm fungi research allowed the assessment of several other aspects of fungal ecology that are essential for studying fungal communities and their biological patterns in the ecosystems. Subjects such as vertical and horizontal distribution (Hyde and Sarma, 2006), fungal succession (Yanna *et al*., 2001a, 2002; Pilantanapak *et al*., 2005) and fungal co--occurrence (Sarma and Hyde, 2018) are frequently assessed on palmicolous fungal assemblages due to their abundance, diversity and patterns on the hosts sampled. This clearly shows a close association of palm fungi with palms hosts, insomuch as they are considered to be good biogeographical indicators (Taylor *et al*., 2000), playing a key role in biodiversity surveys.

4. Palm fungi, the major cause of palm tree leaf spots and leaf blights

As in many other plants, three ecological groups of microfungi are found on palms – endophytes, saprophytes and pathogens (Hyde, 1995h). While endophytes and saprophytes have been intensively studied, relatively few studies on palmicolous fungi have addressed plant pathogens. Additionally, the great majority of these studies focused mostly on the host perspective, consisting more of phytosanitary measures rather than ecological and/or descriptive taxonomy studies of the phytopathogenic agents (Broschat *et al*., 2014, 2015; Elliott, 2004, 2017, 2018a–g, 2019; Yu and Elliott, 2019). The adverse implications of fungal pathogens to quarantine and the ornamental palm industry are long known (Forsberg, 1987), which is a glaring example of important research that has not been carried out.

Although a great diversity of ascomycetes is consistently found associated with palms in the tropics, most are not pathogenic to palms (Fröhlich and Hyde, 2000; Hyde *et al*., 2000; Taylor and Hyde, 2003). Very few fungal pathogens of palm trees are lethal, and the vast majority are responsible for minor damage that when well managed are easily controlled. Common fungal diseases found on ornamental palms include the following: lethal or potentially lethal diseases, such as Fusarium wilt (caused mainly by *Fusarium oxysporum*), Ganoderma root and butt rot/basal stem rot (caused by *Ganoderma* spp.), Gliocladium blight/pink rot (caused by *Nalanthamala vermoesenii*, formerly *Gliocladium vermoeseni*) and Thielaviopsis trunk rot (caused by *Thielaviopsis paradoxa*) and other *Thielaviopsis* diseases; and minor damage diseases, such as several leaf spots and leaf blights, petiole and rachis blights, as well as the diamond scale (caused by *Phaeochoropsis neowashingtoniae*) and other tar spot diseases (Hyde and Cannon, 1999; Broschat *et al*., 2014, 2015; Elliott, 2004, 2017, 2018a–g, 2019; Yu and Elliott, 2019).

In Portugal, some biotic diseases in ornamental palms have been identified since 1998 following studies in order to gain knowledge on phytosanitary problems that depreciate plants in green spaces. Among these, the common fungal diseases reported are the pink rot and Thielaviopsis trunk rot (Ramos *et al*., 2013, 2015). Currently, except for these scattered studies, almost nothing is known about fungal diversity on palm trees in Portugal. In this context, this dissertation intends to gain knowledge on the subject focusing on palmicolous fungi associated with foliar lesions as a starting point.

Leaf spotting palmicolous fungi

The terms “leaf spot” and “leaf blight” are broadly used in the literature to describe a wide variety of different symptoms of plant leaf diseases, which sometimes can be confusing and misleading. In this dissertation, the terms are defined according to Elliott (2018a). Leaf spots are usually rather definite diseased leaf areas that “initially start as small, water-soaked lesions that then turn various shades of yellow, gray, reddish-brown, brown or black” and “are usually surrounded by a halo or ring of tissue that is a different color”. The difference between a leaf spot and a leaf blight depends on the degree of damage to the leaf blade. Leaf blights are often the consequence of the merging of numerous leaf spots that grow up to form irregular blotched areas of diseased tissue. Thus, as pointed out by Elliott (2018a), “as long as the spots are discretely separated from each other by green tissue, the disease is referred to as a spot”, while leaf blights are generally larger diseased areas and more irregularly shaped.

Most leaf spots and leaf blights of palm are caused by fungi and are ubiquitous in palms in production and in the landscape (Broschat *et al*., 2014; Elliot, 2018). However, most of them are more problematic in young palms (Forsberg, 1985). Numerous fungal pathogens can be associated with these diseases, but their symptoms are relatively similar, insomuch that usually no specific name can be allocated to a particular foliar symptom until after the pathogen is determined (Broschat *et al*., 2015). Potential fungal causes of palm leaf spots and leaf blights with a wide host range, both within the *Arecaceae* and other plant families, include the following genera: *Alternaria*, *Annellophora*, *Bipolaris*, *Botryosphaeria*, *Botrytis*, *Calonectria*, *Cercospora*, *Colletotrichum*, *Curvularia*, *Exserohilum*, *Fusarium*, *Graphiola*, *Gloeosporium*, *Nalanthamala*, *Pestalotiopsis*, *Phaeotrichoconis*, *Phyllachora*, *Pseudocercospora*, *Sclerotium*, *Seiridium* and *Stigmina* (Broschat *et al*., 2014, 2015; Elliott, 2018a). Although the complete host range for each of these pathogens is unknown, it is assumed to be broad within palm tree species and it is quite likely that every palm species is susceptible to at least one of them (Elliott, 2018a).

There are a few reports scattered throughout the literature that explore the assemblage of fungi associated with leaf diseases of palms mostly following a descriptive taxonomic approach. Hyde and his co-workers were one of the first to realize that these fungi are an important assemblage in palm leaf diseases that were understudied. Thus, following a commentary on the diseases of palms by Chase and Broschat (1991), later revised as a compendium by Elliott (2004), a remarkable diversity of fungi associated with leaf diseases of palms have been reported. This included some new species of *Capitorostrum*, *Oxydothis*, *Astrosphaeriella*, *Phyllosticta*, *Myelosperma*, *Everhartia*, *Pseudospiropes* and *Cercospora*, and two new genera, *Maculatipalma* and *Maculatifrondis* (Supplementary Table A.2). The leaf spots surveyed in these reports were both from different palm hosts and different regions of the world, namely North Queensland (Fröhlich

and Hyde, 1994, 1995b,c,d; Hyde and Fröhlich, 1995b; Fröhlich *et al*., 1997), Papua New Guinea and Irian Jaya (Hyde and Philemon, 1991; Hyde and Fröhlich, 1995b), Ecuador and South Africa (Hyde *et al*., 1996b), Hong Kong (Yanna *et al*., 2000) and Thailand (Pinnoi *et al*., 2009; To-anun *et al*., 2009; Wulandari *et al*., 2011).

Many other species of leaf spotting palmicolous fungi remain to be described. While most of these studies focused on identifying the cause of the spots or blights, only one study treated these symptoms as possible communities of fungi. In fact, Fröhlich *et al*. (1997) reported the results of a study into the biodiversity of fungi associated with leaf diseases of palms in the rainforests and nurseries of North Queensland, following an integrated approach of culture-dependent methods (i.e. isolations of fungal assemblage associated with the diseased leaf areas). Almost none of these studies were supported by molecular data, which highlights the urgent need to develop studies on the fungi associated with palm leaf spots and leaf blights that can fit in modern methods of fungal diversity studies so the pathogens and their phylogenetic relationships can be placed in a natural taxonomic framework (Konta *et al*., 2017). Subsequently, this will contribute towards the efforts to provide a stable platform for the taxonomy of phytopathogenic fungi that has been the main focus of the Genera of Phytopathogenic Fungi (GOPHY) series of papers (Marin-Felix *et al*., 2018, 2019a,b).

5. Dissertation aim

Palm trees are undoubtedly a key resource in the ornamentation of parks and gardens and as shade trees in Portuguese cities. Anything that diminishes their attractiveness, such as disfiguring leaf diseases, or kills them will negatively affect their aesthetic value. The recent impact of the red palm weevil, *Rhynchophorus ferrugineus*, which has decimated *Phoenix canariensis* in Portugal, has highlighted the fact that pests and diseases introduced onto a host growing outside of its native range can have devastating effects. Considering that relatively few studies on palmicolous fungi have addressed plant pathogens and the almost complete lack of knowledge about fungi on palms in Portugal there is an urgent need to gain knowledge on the assemblage of fungi that are found on palms, especially those associated with diseased trees.

The main aim of the present study was to make a preliminary assessment of the diversity and ecology of fungi associated with palm trees in Portugal, especially those associated with foliar symptoms. A secondary aim was to contribute towards the global effort to discover the missing species of fungi, since palms have proven to be a rich source of new fungal taxa. This information is important, firstly to Portugal's gardening and landscaping industry and plant disease control, since it will contribute to efforts to prevent the entry of plant diseases into the country and to identify possible threats. It will also contribute to the base of knowledge on fungal, and general biodiversity surveys. The study of microfungi associated with palm leaf spots and leaf blights in Portugal is pioneer and represents an innovation in the fundamental knowledge research.

The present dissertation is structured into two parts.

Part I explores the diversity of the fungal assemblage associated with the leaf spots and leaf blights surveyed and assesses ecological traits that can be evaluated at this level. For this purpose, classical and molecular methods for discriminating and monitoring taxa, including isolation, morphological observation and DNA fingerprinting with csM13 and $(GTG)_5$ primers, and analyses for assessing fungal diversity, including calculation of diversity indices, assessment of genera-abundance distribution and genera accumulation curves, were applied. Five questions arose as the main objectives of this part: **(a)** what fungal genera are found associated with foliar lesions on palm trees in Portugal? **(b)** are different fungal genera found on different host species of palm trees with foliar lesions? **(c)** are different fungal genera found in different parishes where palm trees with foliar lesions were surveyed? **(d)** how many fungal genera can co-exist in a single foliar lesion? Is there any association between fungal community and foliar lesions type? **(e)** what degree of genetic diversity exists in the main fungal genera found on palm trees with foliar lesions in Portugal?

Part II provides in-depth analyses of some interesting isolates to determine if they account for some of the undescribed global mycota. For this purpose, currently accepted barcodes were sequenced and used to establish phylogenetic relationships between the new isolates and extant taxa.

PART I

Microfungal communities of palm foliar lesions: from biodiversity to ecological observations

1. Introduction

Fungi play a key role in ecosystems, not only as decomposers essential in nutrient cycles, but also as symbionts and pathogens. Thus, it is of interest to measure and monitor their biodiversity and to study their ecology. Tang *et al*. (2006) pointed out that the driving force for fungal diversity studies outcomes form "the need for knowledge on their ecological functioning, evolutionary relationships, physiological and biochemical properties, and biotechnological and pharmaceutical potential". Only a few fungal species are presently utilized in biotechnological processes or in the production of novel compounds, which implies that there is a huge potential for their industrial exploitation (Hyde *et al*., 2019b). Several data sets have shown that a more complete inventory of microfungi from various ecological niches need to be developed to build up a representative collection of these organisms for future research, society and prosperity (Hyde *et al*., 2007). Hyde *et al*. (2019) outlined an interesting diagram showing the potential use of fungi in biotechnology and this starts with the basic biodiversity research. Modern Mycology is aware that biodiversity research is a key element and an essential tool to accomplish any kind of applied research that in turn will lead to commercialized products.

In the following subsections a methodological and data analysis introduction is presented for some of the methods used throughout the development of the present approach to assess the characterization of the fungal communities surveyed.

1.1. Biodiversity measures: species richness and species evenness

Diversity is a measure of the complexity of structure in an ecological community (Zak and Willig, 2004). It comprises two distinct attributes that are evaluated by quantitative expression of community structure (biodiversity measures), namely species richness (S) – number of species of a given taxon – and species evenness or equitableness (E) – how similar species are in their abundances (Clifford and Stephenson, 1975; Magurran, 2004). Given that species richness is highly dependent on the collection effort, S is of limited value as a comparative index. Thus. species richness indices were developed to decrease the effect of this bias (Ludwig and Reynolds, 1988), such as the Margalef species richness index (D_{Mg}) (Margalef, 1958). These are based on the ratio between the species richness and the total number of individuals in the sample (Magurran, 2004). Besides the species richness indices, several nonparametric species richness estimators have been developed, such as Chao1 (S_{Chao1}) (Chao, 1984). These estimators of richness aim to determine the number of species that have yet to be discovered in the studies' sampling context (Magurran, 2004; Schmit and Lodge, 2005).

1.2. Diversity indices: Shannon and Simpson indices of diversity

Many indices are available in literature for estimating biodiversity measures, but since each index highlights different diversity components no unified diversity index is available (Tang *et al*., 2006). Two of most common diversity indices are the Simpson index of diversity (D) and the Shannon index of diversity (H').

Simpson index of diversity (D) (Simpson, 1949) was the first one to be used in ecology and it is referred to as a dominance measure since it is strongly affected by the abundance of the most common species (Zak and Willig, 2004). As a measure, D reflects the probability of randomly choosing two individuals that belong to the same species (Kim *et al*., 2017). It varies from 0 to 1 and increases as the diversity decreases, so to ensure that the index increases with increasing diversity the reciprocal or complement form of D (1/D or 1 – D) is usually presented (Magurran, 2004).

Shannon index of diversity (H') (Shannon, 1948) is the most widely used measure of diversity in community ecology and represents a measure of the average degree of uncertainty in predicting the specific identity of an individual randomly chosen from a collection of S species and N individuals (Zak and Willig, 2004). Important characteristics of this diversity index are related to the fact that average uncertainty will increase as the numbers of species increases and as the distribution of individuals among species becomes more even (Magurran, 2004).

Both diversity indices have specific biases. While Shannon index of diversity sets a greater weight on species richness, Simpson index of diversity places a greater weight on species evenness (Magurran, 2004; Kim *et al.*, 2017). Each diversity index has a corresponding evenness index that measure the degree to which a certain community displays the maximal diversity possible, given the observed richness (Zak and Willig, 2004). Pielou (1966) proposed an evenness index (J') based on the Shannon index of diversity. The quantity J' expresses the observed diversity (H') as a proportion of the maximum possible diversity (H'_{max}), insomuch that it could be used as a measure of entropy in the distribution of individuals among the species (Zar, 2010).

1.3. Genera-abundance distribution and collector's effort curve

Diversity indices are often of limited use since they compress the data to a single value that little expresses about the species abundance in the ecological community (Zak and Willig, 2004). In this sense, several other strategies have been developed to evaluate the diversity of the ecological community, such as species-abundance distributions and collector's effort curves.

Species-abundance distributions were developed by Fisher *et al.* (1943) in order to examine how the diversity and the structural organization of an ecological community are related. This concept relies in the fact that a characteristic pattern arises when the number of species and their relative abundances within a community are plotted (Zak and Willig, 2004). Thus, the species-abundance distributions are models used to describe the distribution of commonness and rarity in an ecological community (Su, 2018) and their assessment is "a major steppingstone to understanding communities in general" (McGill *et al*., 2007), since they provide the most complete assessment of diversity (Magurran, 2004).

One of the oldest and most common analysis of diversity data is the construction of a species--accumulation curve, also known as collector's effort curve. This is constructed by plotting the cumulative number of species found against a pertinent measure of the effort used in finding them (Magurran, 2004; Schmit and Lodge, 2005). Collector's effort curve is often used as a tool to evaluate the sampling effort or to make comparisons between the diversity of different sampling sites (Magurran, 2004).

1.4. Genomic discrimination and clustering: MSP-PCR

Morphological traits and cultural characteristics have been often very difficult characters to use as exclusive tools in the identification, differentiation and classification of species and strains of filamentous fungi. Subsequently, additional techniques, such as molecular markers, have been successfully used to overcome these problems (Meyer *et al*., 1993a; Rodriguez *et al*., 2004).

Genomic fingerprinting is a molecular method frequently used for microbial genotypic characterization due to its reproducibility and highly discriminatory power (Jeffreys *et al*., 1985; Rademaker and de Bruijn, 1997). This methodology comprises a vast range of DNA-based techniques that provide practical markers, as a result of DNA polymorphisms, for molecular typing (Weising *et al*., 1995). These methods are commonly used as tools in fungal taxonomy, since they allow the discrimination of isolates from intrageneric to strain levels (Soll, 2000). One broadly used class of those methods is the polymerase chain reaction (PCR)--based genomic fingerprinting, such as the microsatellite/minisatellite primed (MSP)-PCR, which analyse the whole genome. This is a good alternative to methods that rely on specifically targeted primers and have been shown to be relatively robust and discriminatory (Olive and Bean, 1999).

MSP-PCR is a genomic fingerprinting technique that uses small sized primers complementary to microsatellites/minisatellites sequences, i.e., ubiquitous tandem repeats of small DNA motifs of 1–5/10–60 bp long present in several copies across the genomes (Lieckfeldt *et al*., 1993; Meyer and Mitchell, 1995; Vogel and Scolnik, 1997). These small sized primers generate DNA fingerprintings that are useful for discriminating between fungal isolates. The primers csM13, the core sequence of the wild-type phage M13, which is specific to minisatellite DNA sequences, and $(GTG)_5$, which are specific to simple repetitive DNA microsatellite sequences, are examples of commonly used primers in this technique (Meyer *et al*., 1991, 1993b, 1997, 1999, 2001; Alves *et al*., 2007; Ramírez-Castrillón *et al*., 2014).

2. Materials and Methods

A schematic overview of the workflow used in the present work is presented in Supplementary Figure A.1.

2.1. Specimen collection and examination

Diseased ornamental palm leaflets and leaf segments, especially those showing leaf spots or leaf blights, were collected during September and October 2018 from parks, gardens, cityscapes and indoor environments in Oeiras and five Lisbon parishes (Alvalade, Areeiro, Marvila, Parque das Nações and São Vicente) (Figure 2.1). Plant material was transported to the laboratory in paper envelopes on which collection details were noted (namely sample number, location, collector, collection date, host and other notes when needed) and examined as soon as possible for associated fungi. Specimens were air-dried and stored in a cardboard box at room temperature (18–20 ℃). Specimens were examined with a Leica MZ9.5 stereo microscope for observations on lesion morphology and for the presence of fungi. Morphological details of lesions were observed on both adaxial and abaxial surfaces. Lesion shape, margin topography, colour and its evolution over time (when possible), presence or absence of a distinctive border, halo or occasional coalescence, overall distribution on the leaflet and, if applicable, the size were recorded.

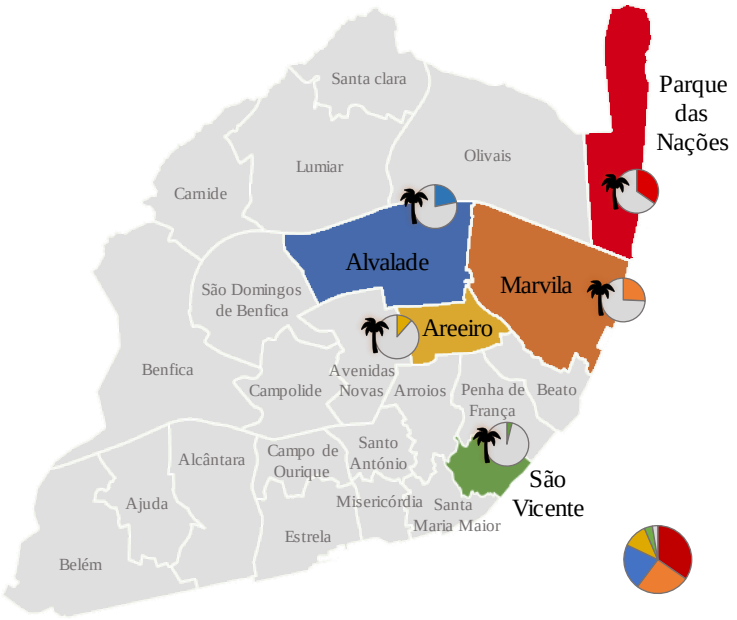

Figure 2.1 Map of Lisbon and respective sampling sites. Each collection site (parish) is highlighted with a different colour and is followed by a pie-chart representing the corresponding percentage of lesions sampled. The samples from Oeiras are not represented on the map but could be assessed on the grey slice present in the general pie-chart on the right, where all the samples are considered with the corresponding parish colour. Map source: adapted from "Lisboa em Números", by Observatório de Luta Contra a Pobreza na Cidade de Lisboa, 2019 (https://observatorio-lisboa.eapn.pt).

2.2. Culture media and growth conditions

Potato Dextrose Agar (PDA) (BIOKAR Diagnostics, France) was prepared at two different concentrations, namely half-strength PDA (1/2 PDA) and quarter-strength PDA (1/4 PDA). Unless stated otherwise, cultures were incubated in ambient light at room temperature (18–20 ℃). To stimulate sporulation, isolates were cultured on 2% water Agar (WA) (BIOKAR Diagnostics, France) with healthy doubled autoclaved (two cycles of 20 min, 121 °C and 1 bar with 48 h between each cycle) *Populus* sp. twigs or palm leaflet pieces on the agar surface. Cultures were incubated at 25 ℃ under black light.

2.3. Fungal isolation

Isolations were made on 1/2 PDA containing 0.05% chloramphenicol (CPDA) to reduce bacterial contamination (Choi *et al.*, 1999) following the isolation flow-chart presented in Figure 2.2. Leaflets and

segments were first examined with a stereomicroscope for the presence of spore-producing structures. If no signs of sporulation were seen the specimens were incubated for 1–3 weeks in a moist chamber and examined daily with the stereomicroscope for signs of sporulation.

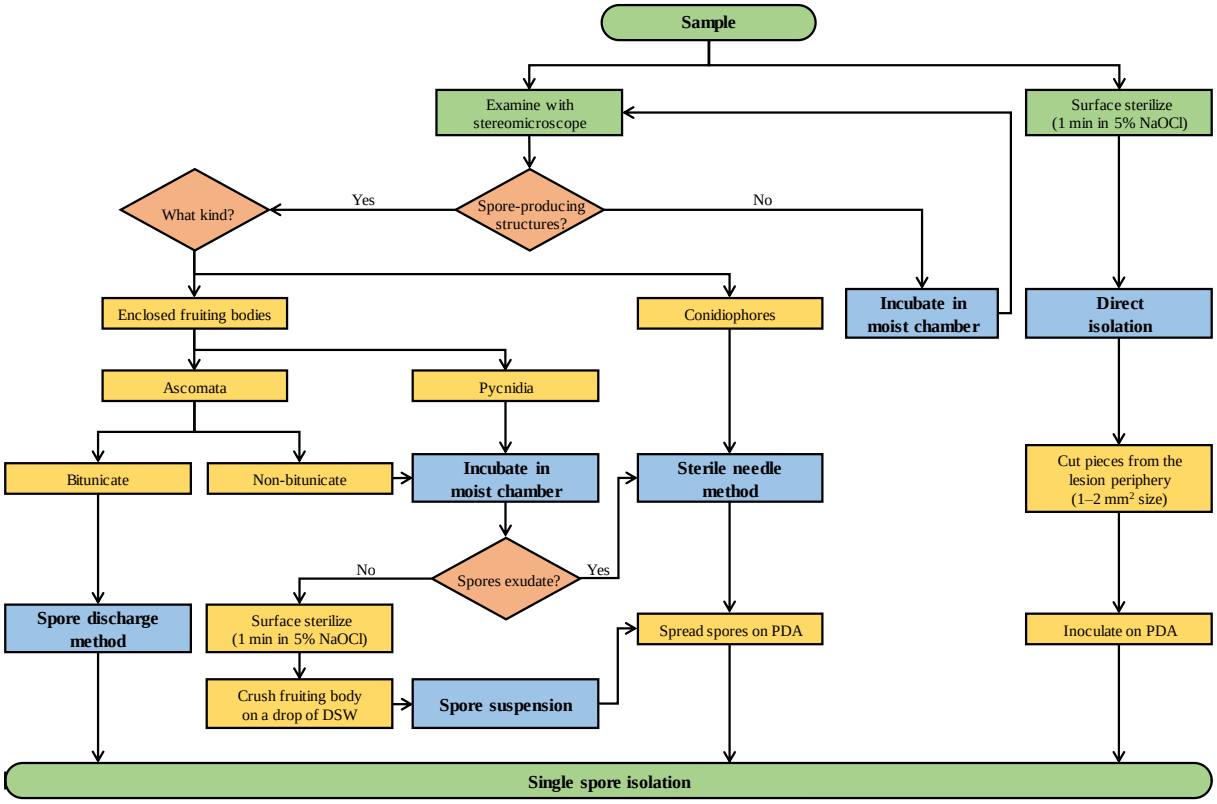

Figure 2.2 Isolation flow-chart. Each colour represents a different step in the isolation process. Green shapes indicate either the beginning or the termination of the process. Orange-diamonds are decision points where the process is split into decisive options. Blue shapes represent the methods of isolation used. Yellow shapes represent methodological steps before an isolation method or the end of the process. DSW = distilled sterile water.

If fertile hyphomycete conidiophores were present, isolations were made by touching the tip of a sterile needle on the conidiophores and spreading the conidial mass on CPDA. After incubation for up to 24 h, 15–20 single germinating conidia were transferred separately onto 1/2 PDA.

If enclosed fruiting bodies were found, they were first examined microscopically to determine whether they were ascomata or conidiomata.

If conidiomata were found, the lesion was incubated in a humid chamber to stimulate spore release. Conidia were then transferred on a sterile needle to plates of CPDA and spread over the agar surface. After a suitable period of incubation, 15–20 single germinating conidia were transferred to fresh plates of 1/2 PDA. If, after 7 days of incubation, no conidia could be seen, the lesion was surface sterilized and the conidioma crushed in a drop of sterile water on a flamed microscope slide. The resulting conidial suspension was diluted 100 times to reduce the chances of contamination (Choi *et al*., 1999) and spread on CPDA, incubated and 15–20 single germinating conidia were transferred separately onto 1/2 PDA.

If bitunicate ascomata were present, a small piece of the lesion bearing ascomata was placed on a drop of sterile water in an upside-down plate of CPDA. Ascospores discharged forcibly and impinged on the agar surface. After 2–5 h single germinating ascospores were transferred to fresh plates of 1/2 PDA.

If non-bitunicate ascomata were present, the isolation methods followed were similar to those used when conidiomata were found.

For all specimens, isolations were also made directly from lesions. Pieces of tissue 1–2 mm^2 were cut from the edge of the lesions, surface sterilized in 5% sodium hypochlorite for 1 min, rinsed in three changes of sterile water and blotted dry on sterile filter paper. Five leaf fragments were plated onto CPDA and

incubated for 24 h until discrete colonies developed (2–7 d). The sterilization efficiency was controlled by impressing the leaf fragments several times (Schulz *et al*., 1998) and inoculating the last sterile water change in a plate of CPDA. A piece of healthy tissue from the same leaflet or segment, subjected to the same sterilization process, was also plated onto CPDA to validate the isolation of fungal records only from diseased tissue. The fungi were subcultured onto 1/2 PDA and single spore isolates established when possible.

2.4. Morphological observation and characterization

The pure cultures on 1/2 PDA were examined periodically to determine culture characteristics as well as the development of microscopic structures. Microscopic structures were mounted in 100% lactic acid and examined by differential interference contrast (DIC) microscopy (Chomnunti *et al*., 2014). Sections 10 µm thick of some reproductive structures were made with a Bright 5040 Rotary Retracting Microtome with a solid state freezer stage (Bright Instruments, UK). Observations on micromorphological features were made with Leica MZ9.5 and Leica DMR microscopes (Leica Microsystems GmbH, Germany) and digital images were recorded with Leica DFC300 and Leica DFC320 cameras (Leica Microsystems GmbH, Germany), respectively. Measurements were made with the measurement module of the Leica IM500 Image Management System (Leica Microsystems GmbH, Germany). Mean, standard deviation (SD) and 95% confidence intervals were calculated from measurements of 50 structures, unless stated otherwise with n = total of measured structures. Measurements are given as minimum and maximum dimensions with mean and SD in parenthesis. Infrequent measurements are also given in parenthesis along with the minimum and maximum dimensions. Photoplates were prepared with Adobe Photoshop CS6 (Adobe, USA). Isolates were identified to genus level when possible by reference to the available literature (e.g. Sutton, 1980; Seifert *et al*., 2011a; Wijayawardene *et al*., 2016).

2.5. Culture storage and preservation

Isolates were stored on 1/4 PDA slants about 2 cm in its widest part in 5 ml graduated microtubes and kept at 4 °C, and at room temperature after being covered with 2 ml of sterile mineral oil.

2.6. DNA extraction

Genomic DNA (gDNA) was extracted from cultures of all isolates following a modified and optimized version of the guanidium thiocyanate method described by Pitcher *et al*. (1989). The isolates were grown on PDA in darkness at 20 °C until a suitable amount of mycelium growth was observed. The mycelium was then scraped off and collected in 2 ml microtubes with 100 µl of autoclaved glass microspheres (425–600 µm diam) and 250 µl of lysis buffer (250 mM NaCl, 50 mM Tris, 50 mM EDTA, 0.3% (w/v) SDS, pH 8.0). The tubes were incubated on ice for 10 min, vortexed three to six times for 2 min at maximum velocity, incubated for 30 min at 65 °C and revortexed for 2 min at maximum velocity. If necessary, a pellet pestle was used to assist in breaking the cell walls. Two consecutive steps of incubation on ice for 10 min were performed, the first upon the addition of 250 µl of GES reagent (5 M guanidium thiocyanate, 100 mM EDTA, 0.5% (v/v) sarkosyl, pH 8.0), which was mixed by inversion, and the second after the addition of 250 µl of cold 10 M ammonium acetate. To separate organic and aqueous phases, 1 ml of chloroform:isoamyl alcohol (24:1) (v/v) was added, mixed by vigorous agitation and then centrifuged at 14 000 rpm for 20 min. The aqueous phase was transferred to a new 1.5 ml tube and the nucleic acids precipitated by the addition of an equal volume of cold absolute isopropanol and mixed by inversion. The tubes were centrifuged again at 14 000 rpm for 20 min, the supernatant discarded and the pellets washed with 1 ml of cold 70% (v/v) ethanol. After a further centrifugation at 14 000 rpm for 20 min, the supernatant was discarded and the pellets dried at room temperature with the tubes open in an inverted position for 5–10 min. After ensuring that all ethanol was removed, the pellets were dissolved in 100 µl of TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0) and stored at 4 °C.

Quality and quantity of the gDNA were evaluated by agarose gel electrophoresis. Thus, 5 µl of each DNA extract were submitted to electrophoresis in a 0.8% (w/v) agarose (Invitrogen, UK) gel, with 0.5× TBE buffer (40 mM Tris, 45 mM Boric acid, 1 mM EDTA, pH 8.3) and a constant voltage of 5.6 V cm^{-1} for

1 h. The gel was stained with 2.5 µg ml^{-1} ethidium bromide solution, visualized with an Alliance 4.7 UV transilluminator (UVITEC Cambridge, UK) and the image recorded with Alliance software version 15.15 (UVITEC Cambridge, UK). The molecular weight marker used was the 1 kb Plus DNA Ladder (Invitrogen, UK). gDNA concentrations were estimated using ImageJ software version 1.52a (Schneider *et al.*, 2012).

2.7. MSP-PCR

MSP-PCR profiles were generated following a modified version of the protocol of Ramírez-Castrillón *et al.* (2014), using csM13 (5' – GAGGGTGGCGGTTCT – 3') (Vassart *et al.*, 1987; Ryskov *et al.*, 1988) and $(GTG)_5$ (5' – GTGGTGGTGGTGGTG – 3') (Walmsley *et al.*, 1989; Lieckfeldt *et al.*, 1993) primers. Both PCR-fingerprintings were performed in a final volume of 25 µl per reaction, containing 1× PCR buffer (Invitrogen, UK), 3 mM $MgCl_2$, 25 pmol of the respective primer, 0.2 mM of each dNTP, 1 U of Taq DNA Polymerase (Invitrogen, UK) and 50 ng of template DNA. Amplification was performed in a TGradient Thermocycler (Biometra, Germany), with the following cycling conditions: initial denaturation at 95 ℃ for 5 min, followed by 40 cycles of denaturation at 95 ℃ for 1 min, annealing at 60 ℃ for 2 min and elongation at 72 ℃ for 2 min, and a final elongation step at 72 ℃ for 5 min. The amplicons were separated by agarose gel electrophoresis, along with the 1 kb Plus DNA Ladder (Invitrogen, UK). Subsequently, 5 µl of reaction mixture was subjected to electrophoresis in 1% (w/v) agarose (Invitrogen, UK) gel, with 0.5× TBE buffer (40 mM Tris, 45 mM boric acid, 1 mM EDTA, pH 8.3) and a constant voltage of 3.4 V cm^{-1} for 5 h. The gel was stained and visualized as described previously (subsection 2.6).

Isolates were clustered based on their csM13- and $(GTG)_5$-PCR profiles in a consensus dendrogram built with BioNumerics software version 6.6 (Applied Maths, Belgium), using Pearson's correlation coefficient to generate the similarity matrix and Unweighted Pair Group Method with Arithmetic Mean (UPGMA) as the clustering method. The reproducibility cut-off level was calculated as the mean value of the reproducibility obtained for each primer independently. For this purpose, and for each primer, 10% of the isolates were chosen randomly and their profiles redone (Sneath and Johnson, 1972). A dendrogram constructed based on these duplicates was used to estimate the reproducibility cut-off level and to calculate the optimization and curve smoothing parameters, 1.5% and 2.5% (for csM13) and 1.5% and 1.25% (for $[GTG]_5$) respectively, that better paired the repeats for each primer. A conservative estimate of the reproducibility cut-off level was established at 95%, above which isolates cannot be distinguished using this technique.

2.8. Biodiversity and ecological observations data analysis

Although biodiversity measures are usually used at species level, in this dissertation they were used at genus level, since most of the isolates were not identified to species level with modern Mycology methods (i.e. sequence of appropriate gene barcodes) (Jeewon and Hyde, 2016). Considering that morphological observation is usually sufficient to identify the isolates to genus, to diminish the report of uncertain results all biodiversity analyses were done at genus level.

2.8.1. Percentage abundance and frequency of occurrence calculation

Fungal records are presented in terms of their percentage of frequency of occurrence, frequency of co-occurrence and abundance of occurrence. Frequency of occurrence (FO) was computed following equation (2.1).

$$\mathrm{FO} = \frac{\text{number of samples on which a given taxon occurred}}{\text{total number of samples examined}} \times 100 \qquad (2.1)$$

Frequency of co-occurrence (FCO) was computed following equation (2.2).

$$\mathrm{FCO} = \frac{\text{number of other fungal genera with which a particular fungal genus has co-occurence}}{\text{total number of genera recorded}} \times 100 \qquad (2.2)$$

Abundance of occurrence (AO) was computed following equation (2.3). Based on the abundance of occurrence of different genera they were grouped as very frequent (>10%), frequent (>5–10%), infrequent (>1–5%) or rare (≤1%).

$$\mathrm{AO} = \frac{\text{number of occurrences of a given taxon}}{\text{total number of occurrences of all taxa}} \times 100 \tag{2.3}$$

2.8.2. Diversity indices calculation

Genera diversity of fungal communities was evaluated according to Mangurran (2004) using Simpson index of diversity, Shannon index of diversity and evenness index along with Margalef species richness index and Chao1 species richness estimator assessed for each genus, collection site or palm species, when applicable.

Simpson index of diversity (D) (Simpson, 1949) was computed from equation (2.4).

$$\mathrm{D} = 1 - \sum \frac{n_i(n_i - 1)}{N(N-1)} \tag{2.4}$$

where n_i is the number of individuals (isolates) in the ith genus, and N is the total number of isolates. Simpson index of diversity was computed in its complement form (1 – D) to ensure that the index increases with increasing diversity.

Shannon index of diversity (H') (Shannon, 1948) was computed from equation (2.5).

$$\mathrm{H}' = -\sum (p_i \log p_i) \tag{2.5}$$

where p_i (n_i/N) is the proportional abundance of the ith genus and log is the common log (base 10).

Evenness index (J') (Pielou, 1966) was computed from equation (2.6).

$$\mathrm{J}' = \frac{\mathrm{H}'}{\mathrm{Log\ S}} \tag{2.6}$$

where S is the genera richness (i.e. total number of genera) and log is the common log. Log S is equivalent to $\mathrm{H'}_{max}$, considering equation (2.5) for Shannon index of diversity.

Shannon index of diversity (H') and evenness index (J') were also applied to molecular fingerprinting data, establishing a cluster cut-off at 70% to define groups. In this context n_i is the number of individuals (isolates) in the ith group, p_i is the proportional abundance of the ith group and S is the total number of groups. A specific terminology was adopted to distinguish the application of these indexes (when applied together) to both morphological and molecular data. Shannon index of diversity was referred to as $\mathrm{ID_m}$ (morphological index of diversity) when applied to morphological data and as $\mathrm{ID_g}$ (genetic index of diversity) when applied to molecular fingerprinting data. Evenness index was referred to as $\mathrm{E_m}$ (morphological evenness) when applied to morphological data and as $\mathrm{E_g}$ (genetic evenness) when applied to molecular fingerprinting data.

Margalef species richness index ($\mathrm{D_{Mg}}$) (Margalef, 1958) was computed from equation (2.7).

$$\mathrm{D_{Mg}} = \frac{(\mathrm{S} - 1)}{\mathrm{Ln}\ N} \tag{2.7}$$

where S is the genera richness, N is the total number of isolates and ln is the natural log (base e).

Chao1 species richness estimator ($\mathrm{S_{Chao1}}$) (Chao, 1984) was computed from equation (2.8).

$$\mathrm{S_{Chao1}} = \mathrm{S_{obs}} + \frac{a^2}{2b} \tag{2.8}$$

where S_{obs} is the number of genera observed (equivalent to S), *a* is the number of observed genera represented by a single individual (singletons) and *b* is the number of observed genera represented by two individuals (doubletons).

2.8.3. Colonization and isolation rates calculation

Fungal isolation was evaluated using colonization and isolation rates. Colonization rate (CR) was computed from equation (2.9). Colonization rates are widely used in the literature and are usually expressed as percentages (Taylor *et al*., 1999; Fröhlich *et al*., 2000a).

$$\mathrm{CR} = \frac{\text{total number of samples yielding} \geq 1 \text{ isolate}}{\text{total number of samples in a given collection site/palm species}} \quad (2.9)$$

Isolation rate (IR) was computed from equation (2.10). Isolation rates (number of isolates per sample) were not expressed as percentages and were calculated to demonstrate the degree of multiple colonization from the samples, so it can be used as a measure of fungal richness in a given collection site or palm species.

$$\mathrm{IR} = \frac{\text{total number of isolates yielded in a given collecting site/palm species}}{\text{total number of samples in a given collection site/palm species}} \quad (2.10)$$

2.8.4. Genera-abundance distribution and collector's effort curve

Genera-abundance distribution was constructed to assess the pattern of genera abundances obtained for the leaf spotting fungal community by plotting the abundance of occurrence of each genus in the sample set on a logarithmic scale against genus rank from most to least abundant.

Collector's effort curve was constructed to estimate whether the sampling was thorough by plotting the cumulative number of genera recovered against the number of samples examined (selected at random) for each parish and for the overall sample set.

3. Results and Discussion

3.1. Overall morphological diversity: what fungal genera are there?

A collection of 457 isolates associated with foliar lesions of palms was established. A total of 57 genera were recorded. This included 32 coelomycetes (38% of all records), 19 hyphomycetes (53%), five ascomycetes and one basidiomycete (5%). In addition, 16 isolates (4%) failed to sporulate (Table 3.1, Figure 3.1). An overview of the distribution of all fungal genera recorded is presented in Figure 3.1. Patterns of diversity and abundance can be examined qualitatively by comparing the taxonomic distribution of fungal taxa. The percentage abundance and frequency of occurrence of all collections of all genera are presented in Table 3.2.

Table 3.1 Distribution of fungal records per fungal types. Absolute frequencies of fungal isolates found associated with the foliar lesions of palms are distributed per different fungal types. Ascomycetes and basidiomycetes correspond to those isolates where only the sexual morph was observed. Sterile mycelium corresponds to those isolates where no sporulation was observed.

Type	Number of isolates	Number of genera
Coelomycetes	173	32
Hyphomycetes	243	19
Ascomycetes	24	5
Basidiomycetes	1	1
Sterile mycelium	16	NA*

*NA, not applicable.

The most common taxa were *Alternaria* (21% of all records), *Cladosporium* (12%) and *Phoma* (10%) with at least 40 isolates recorded. Thus, these genera were regarded as very frequent (Figure 3.1, Table 3.2).

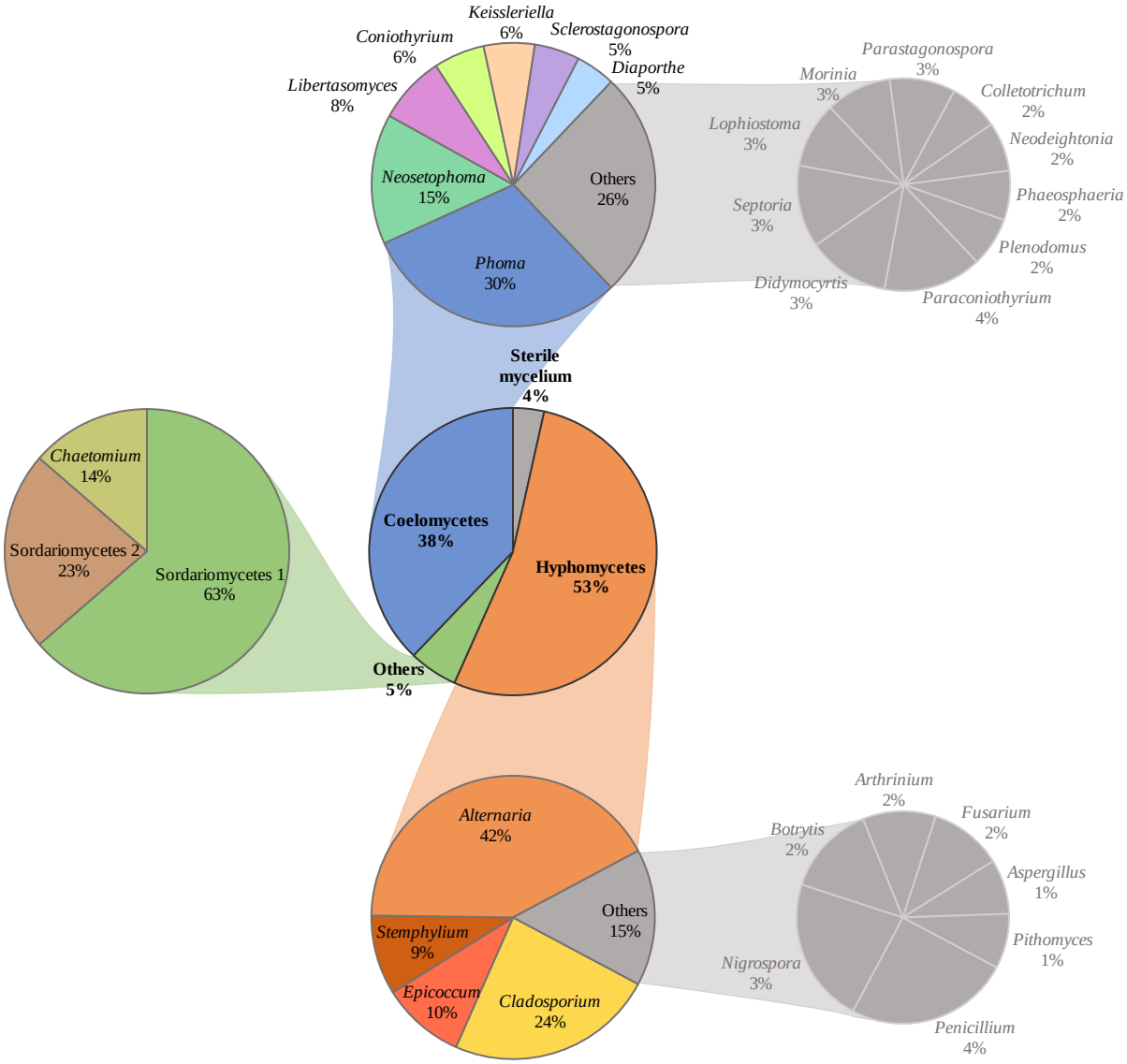

Figure 3.1 Distribution of fungal records per fungal genera. Relative abundance of the main genera of fungal isolates found associated with the foliar lesions of palms examined. Genera with a percentage abundance of occurrence < 0.5% (less than three isolates recorded) were excluded. Colours are according to fungal types and fungal genera.

Only one genus, *Neosetophoma* (5% of all records) can be regarded as frequent, while 15 genera can be regarded as infrequent with an abundance of occurrence ranging from five to just over 20 isolates. The remaining 38 genera were represented by less than five records and can be regarded as rare. Most of them corresponded to coelomycete genera (Table 3.2). A total of 35 fertile isolates remained unidentified to genus level, although it was possible to establish different "morphological groups" based on their culture and micromorphological characters. Thus, 6 hyphomycetes and 5 coelomycetes unnamed genera are presented just as different genera based on their morphological characters. A similar approach was used for the isolates regarded as *Diatrypaceae* genus, *Sordariomycetes* genus 1, *Sordariomycetes* genus 2 and *Teratosphaeriaceae* genus 1, although molecular information has also been used to establish their "morphological groups". Nevertheless, most of these isolates were represented by a single record and account for the rare fungal assemblage of foliar lesions of the palms examined (Table 3.2).

The overall composition of the fungal community associated with the foliar lesions shows a well-defined pattern of dominance by few genera – *Alternaria*, *Cladosporium* and *Phoma* – along with a

remarkable profusion of infrequent and rare genera (Figure 3.1, Table 3.2). More than 66% of the genera recorded have an abundance of occurrence less than 1%. Furthermore, besides the high abundance of occurrence, the dominant genera also showed a high frequency of occurrence, being recorded in almost 50% of the samples analysed (Table 3.2). Thus, *Alternaria*, *Cladosporium* and *Phoma* are not only the genera with the greatest number of isolates, but also the genera that are most frequently found associated with the foliar lesions. Although this study is focused on the biodiversity observations at genus level, the community pattern observed is commonly reported in nature. In fact, the pattern of a few very common species and a wide array of species with only one or two occurrences is common in investigations of plant, animal and fungal diversity and have been reported in studies of both palmicolous endophytes and saprobes (Fröhlich *et al*., 2000a; Pinnoi *et al*., 2006; Pinruan *et al*., 2007). Considering the morphological and molecular fingerprinting characters obtained for each genus, a similar result for community pattern is expected at species level, especially considering the csM13-$(GTG)_5$ clustering profile of *Alternaria* isolates, which is later discussed (subsection 3.5).

This fungal community pattern is well evidenced by the genera-abundance distribution plot (Supplementary Figure A.2), which is best described by a log-series model (May, 1975). The log-series model occurs when one or a few factors control species distribution and when the intervals between arrivals of species into a habitat are random, resulting in only a few taxa becoming dominant (Zak and Willig, 2004). The observed steep slopes in the genera-abundance distribution plot are indicative of an assemblage with high dominance, just as the one recorded on the foliar lesions. Thus, the leaf spotting fungal assemblage of palms could be shaped by few factors concerning the establishment of the symptoms, which should be related with the interaction between the fungal individuals and the plant tissue. Once the symptoms are established by the primary pathogens, the subsequent mycota can arrive regularly and occupy the already damaged tissue with a limited portion of nutrients available. In fact, log-series model predicts that dominant species preempts largest portion of limiting resource (Mangurran, 2004). Consequently, a nonequilibrium assemblage arises, where *Alternaria*, *Cladosporium* and *Phoma* are present with an abundance of occurrence several orders of magnitude higher than the remaining infrequent and rare assemblages. Although the genera-abundance distribution clearly evidences a community with a dominance pattern, any approach for fitting the model was followed, since it is out of the scope of this dissertation. The observed tendency can be expressively changed when isolates are identified at species level. Additionally, the sterile isolates of fungi obtained from the samples should have been sorted into morphological genera groups based on macromorphological and micromorphological characteristics expressed when grown on different media, since when plotting species--abundance distribution, the same taxonomic units must be applied for all ecological units (Zak and Willig, 2004). A biased result is not expected with the identification of sterile mycelium isolates because their abundance of occurrence is remarkably low (Table 3.1).

Different aspects could be related with the dominance of *Alternaria*, *Cladosporium* and *Phoma* (more than 40% of all fungal records) in the community (Table 3.2). These three genera are unlikely to be the main cause of the palm leaves symptoms surveyed. Although they are reported as common fungal genera in leaf spot diseases, including palms (Broschat *et al*., 2014, 2015; Pegg and Manners, 2017; Elliott, 2018a), usually they are observed as opportunistic fungi causing secondary infections. Infection of the plant tissue by primary pathogens may cause changes in the nutrient availability on the leaf surface, which stimulates the already present saprophytic fungi to grow on it and inhabit it as secondary minor pathogens (Abdel-Hafez *et al*., 2015). Secondly, these genera are common fungal saprobes, and consequently could be recorded only as surface contaminants due to an inefficient surface sterilization process (Hyde and Soytong, 2008).

Considering the overall diversity of the fungal communities of foliar lesions, in terms of both genera richness and abundance of occurrence, isolates may be classified into three groups: (a) well-known and important pathogens of foliar lesions, such as most of the coelomycetes recorded; (b) commonly abundant phylloplane fungi which are considered primary saprobes and secondary minor pathogens, such as most of the *Alternaria*, *Cladosporium* and *Phoma* isolates recorded; (c) occasionally occurring fungi that were possibly isolated only as contaminants and could not usually integrate the foliar lesions communities, such as some of the rare occurring genera.

The fungal assemblage associated with the foliar lesions of palms examined have a broad taxonomic distribution, although at least 20 of the 57 genera (35%) are members of the order *Pleosporales*. This includes most of the genera with the highest percentage abundance of occurrence. In fact, most of the isolates recorded were members of the order *Pleosporales*, with a remarkable contribution from the genera *Alternaria* and *Phoma*. Nevertheless, *Cladosporium* arises as an exception, since it was the second most abundant genus in the community, although it is a member of the order *Capnodiales*. The remaining genera are dispersed through a wide range of fungal orders and, especially families, that occur rarely. Recent reports of palmicolous fungi have described a series of *Botryosphaeriales*, including members of the genera *Botryosphaeria*, *Phyllosticta* and *Neodeightonia* (Liu *et al*., 2010, 2012; Wulandari *et al*., 2011) as common fungal genera found on palms. Members of this order, comprising five different genera in the family *Botryosphaeriaceae*, namely *Neodeightonia*, *Fusicoccum*, *Neofusicoccum* and *Diplodia*, and *Phyllostictaceae*, namely *Phyllosticta*, were recorded in the present study. Concerning higher taxa, as expected, *Sordariomycetes* (at least 14 in 57 genera) and *Dothideomycetes* (at least 28 in 57 genera) were the best represented classes found associated with foliar lesions of palms in Portugal.

A pattern has emerged from previous studies of a group of fungi, mainly ascomycetes, consistently associated with palms, which are referred to as palm fungi or palmicolous fungi (Hyde *et al*., 1997c; Fröhlich and Hyde, 2000; Hyde *et al*., 2000; Taylor and Hyde, 2003). Although the most representative group of leaf spotting palmicolous fungi found was the ascomycetes, noteworthy differences in the taxonomy of the foliar lesions' assemblage were found compared to what has been reported. The assemblage of palmicolous fungi associated with foliar lesions in Portugal differs from the assemblages of palmicolous fungi reported in tropical regions. While *Xylariaceae* was found to be the best represented family on tropical palms (Zhang *et al*., 2019), in the present study the best represented families were the *Pleosporaceae* in the hyphomycetes and the *Phaeosphaeriaceae* in the coelomycetes. These results were expected, since climatic influences are one of the factors that shape palmicolous fungal communities (Taylor *et al*., 2000).

Fungal host and family specificity are often reported on palm trees in the tropics (Hyde *et al*., 2007). However, fungi associated with palms in their native habitat are not necessarily the same ones reported when they are moved into temperate regions (Broschat *et al*., 2014). In fact, the assemblage of leaf spotting fungi associated with ornamental palms in Portugal seems to be composed of ubiquitous genera frequently reported as foliar pathogens, including palms, such as *Alternaria*, *Arthrinium*, *Botrytis*, *Colletotrichum*, *Fusarium*, *Neopestalotiopsis*, *Nigrospora*, *Stemphylium*, *Pithomyces*, *Phoma*, *Phyllosticta*, *Epicoccum* and several coelomycete genera. This pattern has already been reported in ecological studies on palmicolous fungi. Taylor *et al*. (2000) demonstrated that while the fungi associated with many palm species in their native habitat were a characteristic and consistent assemblage, especially in the tropics, the fungi associated with these same palms outside of tropical regions were composed of fungi considered to be relatively ubiquitous with a much wider plant family host range. In addition, studies carried out by Hyde *et al.* (2000) on the fungal assemblage of the temperate climate palm *Chamaerops humilis* in Europe showed that more temperate and widespread taxa are recorded, consistent with the results from the present study.

What is the composition of the hyphomycete and coelomycete genera assemblage?

A great diversity of hyphomycete and coelomycete genera were recorded (Figure 3.1, Supplementary Figure A.8, Supplementary Figure A.9), however most genera were represented by a small number of isolates and can be regarded as infrequent or rare (Table 3.2). Although abundance of occurrence of hyphomycete assemblage was higher than coelomycete assemblage (53% to 38%), a different result was recorded in terms of genera richness. Genera richness of coelomycetes was almost twice that hyphomycetes (32 to 19; Table 3.1). Thus, diversity of the coelomycetes was higher compared to that of hyphomycetes, both in terms of genera richness and evenness (Table 3.3). These two components play a key role in shaping the diversity of these fungal assemblages and, subsequently, of all fungal communities associated with palm foliar lesions.

The most representative hyphomycete genera recorded were *Alternaria* (21% of all records), *Cladosporium* (12%), *Epicoccum* (5%) and *Stemphylium* (5%). Thus, the main families of the hyphomycete assemblage associated with foliar lesions of palms were *Pleosporaceae*, *Didymellaceae*, in the order

Pleosporales, and *Cladosporiaceae* in the order *Capnodiales*, although several other orders and families were also recorded with a much smaller number of isolates (Figure 3.1, Table 3.2).

Table 3. 2 Distribution of fungal genera per frequency groups. Genera are grouped based on their abundance of occurrence as very frequent (>10%), frequent (>5–10%), infrequent (>1–5%) or rare (≤1%). Percentage of abundance of occurrence and frequency of occurrence is given next to each genus.

Frequency group	Genera (AO*; FO#)	Frequency group	Genera (AO, FO)
Very frequent	*Alternaria* (21.03; 48.72)	Rare	*Cytospora* (0.22; 1.28)
	Cladosporium (12.04; 44.87)		*Diatrypaceae* genus (0.22; 1.28)
	Phoma (10.28; 41.03)		*Diplodia* (0.22; 1.28)
Frequent	*Neosetophoma* (5.03; 15.38)		*Foliophoma* (0.22; 1.28)
Infrequent	*Botrytis* (1.09; 6.41)		*Fusarium* (0.88; 3.85)
	Coniothyrium (1.97; 6.41)		*Fusicoccum* (0.44; 2.56)
	Diaporthe (1.53; 7.69)		*Graphiola* (0.22; 1.28)
	Didymocyrtis (1.09; 5.13)		Hyphomycete genus 1 (0.44; 2.56)
	Epicoccum (4.81; 16.67)		Hyphomycete genus 2 (0.22; 1.28)
	Keissleriella (1.97; 6.41)		Hyphomycete genus 3 (0.66; 3.85)
	Libertasomyces (2.63; 8.97)		Hyphomycete genus 4 (0.44; 2.56)
	Nigrospora (1.75; 6.41)		Hyphomycete genus 5 (0.22; 1.28)
	Paraconiothyrium (1.31; 3.85)		Hyphomycete genus 6 (0.22; 1.28)
	Penicillium (1.97; 10.26)		*Lophiostoma* (0.88; 5.13)
	Sclerostagonospora (1.75; 8.97)		*Monilia* (0.22; 1.28)
	Septoria (1.09; 6.41)		*Morinia* (0.88; 5.13)
	Sordariomycetes genus 1 (3.06; 10.26)		*Neodeightonia* (0.66; 3.85)
	Sordariomycetes genus 2 (1.09; 5.13)		*Neofusicoccum* (0.44; 2.56)
	Stemphylium (4.60; 17.95)		*Neopestalotiopsis* (0.44; 2.56)
Rare	*Arthrinium* (0.88; 5.13)		*Parastagonospora* (0.88; 2.56)
	Ascochyta (0.22; 1.28)		*Phaeosphaeria* (0.66; 2.56)
	Aspergillus (0.66; 3.85)		*Phyllosticta* (0.22; 1.28)
	Bartalinia (0.22; 1.28)		*Pithomyces* (0.66; 1.28)
	Chaetomium (0.66; 2.56)		*Plenodomus* (0.66; 3.85)
	Coelomycete genus 1 (0.22; 1.28)		*Pseudoconiothyrium* (0.22; 1.28)
	Coelomycete genus 2 (0.22; 1.28)		*Stachybotrys* (0.22; 1.28)
	Coelomycete genus 3 (0.22; 1.28)		*Stagonosporopsis* (0.22; 1.28)
	Coelomycete genus 4 (0.22; 1.28)		*Teratosphaeriaceae* genus 1 (0.22; 1.28)
	Colletotrichum (0.66; 2.56)	Total number of genera = 57	
		Total number of identified genera = 43	
		Total number of unidentified genera (“morphogroups”) = 14	
		Total number of isolates = 457	

*AO, abundance of occurrence (%).
#FO, frequency of occurrence (%).

The most representative genera of coelomycetes recorded were *Phoma* (10% of all records) and *Neosetophoma* (5%). Thus, the main families of the coelomycete assemblage found with foliar lesions of palms were *Didymellaceae* and *Phaeosphaeriaceae* in the order *Pleosporales*. Most of coelomycete genera recorded were represented by a small number of isolates and, although a vast array of families was recorded, most of the isolates fit within the order *Pleosporales*. Nevertheless, members of the order *Botryosphaeriales* were also common (Figure 3.1, Table 3.2).

Coelomycete assemblage had the highest diversity index, with a remarkably high evenness index (Table 3.3). This reflects the fact that there were fairly few dominant genera and a high proportion of infrequently collected genera. Although hyphomycete assemblage had a considerably high diversity index, it had an evenness index just over 0.6 (Table 3.3). This reflects the high dominance of *Alternaria* and *Cladosporium* genera, which together represent more than 60% of the hyphomycete assemblage, while in the coelomycete assemblage *Phoma* dominance had less than 30% the total. The fact that Simpson index of diversity was very similar in both assemblages (Table 3.1) also expresses this dominance pattern. In fact, this diversity index gives more weight to the more abundant species in a sample and the addition of rare species cause only small changes (Mangurran, 2004). Consequently, the dominance of *Phoma* in the coelomycete assemblage reduces its diversity and Simpson index of diversity becomes relatively blind to the vast array of infrequent genera. Thus, diversity patterns are more expressive in the Shannon index of diversity value. It must be noted that both assemblages have a substantial number of infrequently recorded genera, but the relative proportion of these genera to the abundance of the dominant ones makes the hyphomycete assemblage much less even.

Table 3.3 Biodiversity measures of hyphomycete and coelomycete assemblages. Simpson index of diversity (D), Shannon index of diversity (H') and evenness index (J') were computed considering the number of genera and the number of isolates recorded in both hyphomycete and coelomycete assemblages.

Index of diversity	Coelomycetes	Hyphomycetes
D	0.89	0.78
H'	1.20	0.84
J'	0.80	0.66

Considering the overall distribution pattern of the community of palmicolous leaf spotting fungi recorded, which follows a log-series model (Supplementary Figure A.2), the hyphomycete assemblage contributes more to the steeper slopes expressing the high dominance, while the coelomycete assemblage contributes more to the shallower slopes expressing the profusion of the infrequent mycota. The coelomycete records were one of the differences detected by Taylor *et al*. (2000) concerning the biogeographical distribution of microfungi associated with palms from tropical and temperate habitats. Besides the few common palm fungi encountered in temperate regions, coelomycetes appeared to be more abundant. The present results confirm Taylor's findings, since the high abundance of hyphomycetes is probably biased due to the records of *Alternaria* and *Cladosporium*. Excluding the dominant mycota in both assemblages, coelomycetes present a higher abundance compared to the hyphomycetes (49% to 35%), so coelomycetes are expected to be more abundant in the fungal mycota associated with palm foliar lesions in Portugal. The ratio between relative abundances of coelomycetes and hyphomycetes found in this dissertation is similar to that found by Taylor *et al*. (2000) for temperate regions (1.4 and 1.3, respectively). This is particularly interesting, considering that these ratios were obtained from relatively different contexts: (a) while the present study used different palm host species as ornamentals in a country with temperate climate (Chazarra *et al*., 2011), Taylor's conclusions were based only on *Trachycarpus fortunei* as a model to study biodiversity on palms from temperate regions; (b) while the present study analysed fungal communities living on palm foliar lesions, Taylor studied fungal communities living on different decaying palm parts; (c) while in the present study collections were made during the end of the dry season (ending of the summer of 2018) (Chazarra *et al*., 2011), Taylor's study included collections made during the wet season.

3.2. Fungal assemblages and host species: are different hosts harbouring different fungal genera communities?

A total of 50 trees in eight different palm species were examined for associated foliar lesions. These included one *Chamaedorea elegans*, two *Phoenix reclinata*, three *Washingtonia filifera*, five *Trachycarpus fortunei*, six *Dypsis lutescens*, six *Phoenix dactylifera*, 13 *Chamaerops humilis* and 14 *Phoenix canariensis*. *Chamaedorea elegans*, *Phoenix reclinata* and *Washingtonia filifera* were excluded from the biodiversity

analyses, since the number of trees examined and, thus, the number of foliar lesions collected (less than five) were too small for any supported trends observation.

All palm host species examined were found to support a considerable wealth of diseased foliar tissue. In general, the greater the number of trees examined, the greater the number of different foliar lesions collected. As stated by Elliott (2018a), all palms should be considered hosts for leaf spots and leaf blights. Nevertheless, in proportion to the number of trees examined, *Phoenix dactylifera*, *Phoenix canariensis* and *Chamaerops humilis* presented a higher number of different foliar lesions than *Trachycarpus fortunei* and *Dypsis lutescens* (Figure 3.2 A, B).

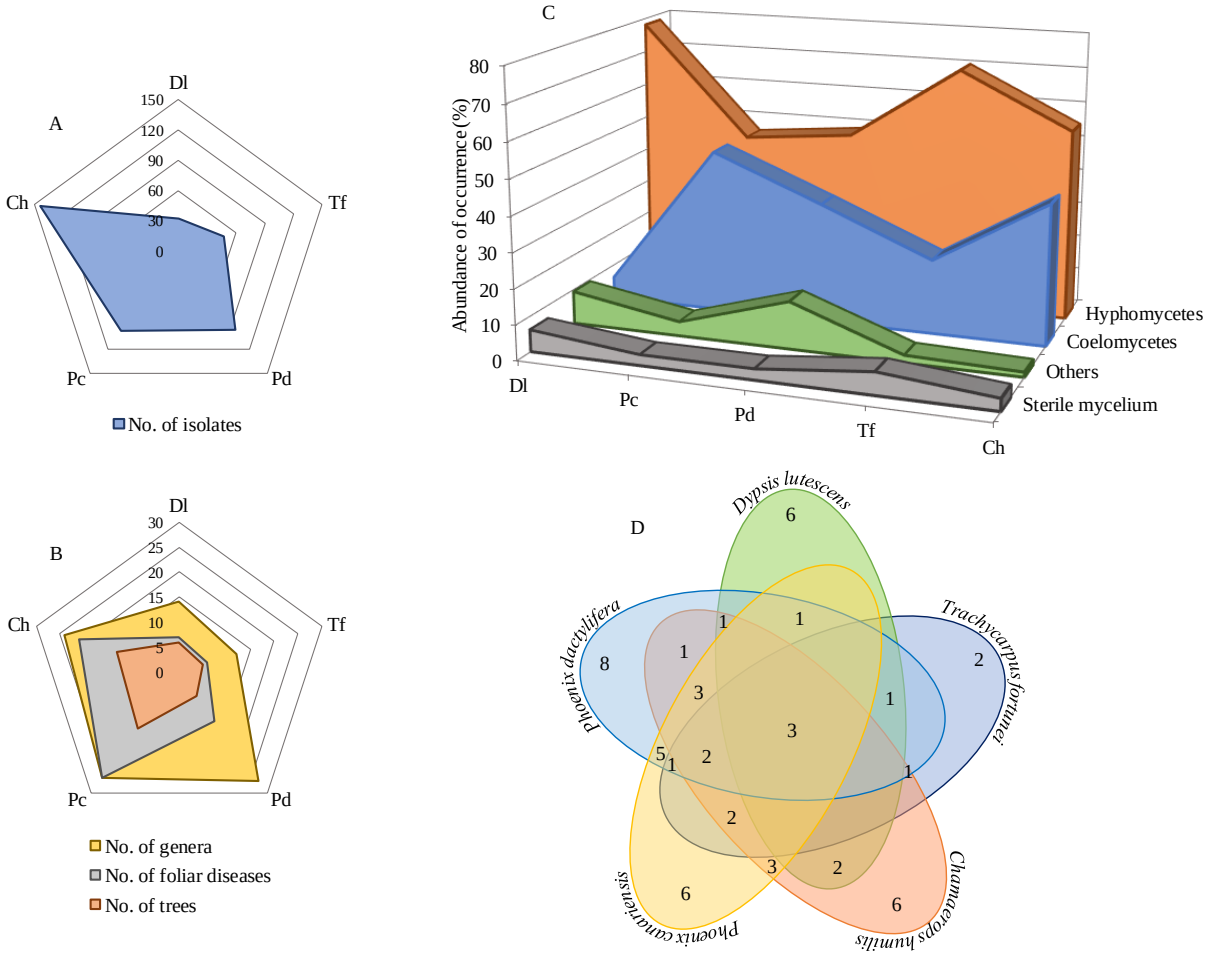

Figure 3.2 General biodiversity patterns of fungal records per host species. **A, B**. Graphical representation of the absolute frequency of isolates and genera yielded, and foliar lesions (samples) and trees examined for each host species. The absolute frequency of isolates was plotted separately due to marked differences in scale values that would impair data visualization if all variables were plotted together. **C**. Percentage abundance of occurrence of each fungal type for each host species. **D**. Venn diagram of genera nicheness by host species. Host species with an absolute frequency of less than five foliar lesions examined were excluded due to sampling effort bias. In C colours are according to fungal types. In D colours are according to host species. Dl = *Dypsis lutescens*, Tf = *Trachycarpus fortunei*, Pd = *Phoenix dactylifera*, Pc = *Phoenix canariensis*, Ch = *Chamaerops humilis*.

Although this could be biased due to the sampling effort and the age of the trees examined, the environment from where these trees were sampled may also have influenced their degree of infection. *Trachycarpus fortunei* and *Dypsis lutescens* were always found in private gardens or indoor environments. Naturally, these are less aggressive than outdoor environments where weather conditions and its changes can influence leaf health and facilitate fungal infections. Climatic events, such as wind, frost, rain or drought, may cause injuries on the leaf blades. These, in turn, can render the palm trees more vulnerable and increase their susceptibility to fungal infections by providing physical entries, such as already verified in some fungal outbreaks (Kim *et al*., 2015). Additionally, such environmental conditions, especially temperature, humidity and rainfall, also widely affect fungal spore production, dispersal, germination and infection (Agrios, 2005;

Pegg and Manners, 2017), favoring foliar lesions development in outdoor environments. Furthermore, palms in private gardens and indoor environments are usually treated by their owners to maintain their aesthetic value and their function as decorative elements. This often implies the use of disease management strategies that aim to eliminate or prevent leaf spots and leaf blights (Pegg and Manners, 2017).

In general, the greater the number of different foliar lesions collected, the greater the number of fungal isolates and fungal genera recorded. *Phoenix dactylifera* arises as an exception, since it was one of the host species with a smaller number of trees and foliar symptoms examined but was the host species with the highest genera richness (27 of all genera records), followed by *Phoenix canariensis* and *Chamaerops humilis*. *Chamaerops humilis* was the host species with the highest number of isolates recorded (144 of all fungal records) (Figure 3.2 A, B). The biodiversity pattern in each host species was similar to the pattern observed in the overall community (Supplementary Figure A.2, Supplementary Figure A.3). Thus, a high profusion of infrequent and rare genera was recorded, along with few very frequent genera (Supplementary Figure A.3). These very frequent genera – *Alternaria*, *Cladosporium* and *Phoma* – were the only three of the 57 genera recorded that were found in all host species (Figure 3.2 D). The main difference between the fungal assemblages of different host species is in the composition of infrequent and rare genera, since most of them are represented by one or two isolates contributing for the exclusive genera composition recorded on each host (Supplementary Figure A.3, Figure 3.2 D). The host species with higher number of exclusive genera was *Phoenix dactylifera* (eight of total genera records), followed by *Chamaerops humilis*, *Dypsis lutescens* and *Phoenix canariensis* (each with six exclusive genera). *Trachycarpus fortunei* was the host species with the lowest number of exclusive genera (three of total genera records). *Phoenix canariensis* and *Phoenix dactylifera* were the host species that shared the higher number of fungal genera that occur only in two host species (five of total genera records) (Figure 3.2 D).

Considering that the fungal assemblages associated with foliar lesions are being studied as fungal communities, if outdoor and indoor environments can influence fungal infections, it is expected that differences in biodiversity of fungal assemblages can arise from this. In fact, this could be the reason why *Phoenix dactylifera* was shown to be one of the species with the highest genera richness and number of isolates recorded even though it was one of the species with a smaller number of trees and foliar lesions examined, just such as *Dypsis lutescens* and *Trachycarpus fortunei*. Additionally, the last two were the species with the lowest genera richness and number of isolates. Therefore, outdoor *versus* indoor environments could explain in part the differences found in the degree of foliar infections shown by different palm host species. The potential phytopathogenic microbial load in outdoor environments, such as streets, boulevards and public gardens may be higher, since it can flow through different trees associated with different spore spreading agents, like animals or the wind, increasing the possibility of plant infection. In this sense, *Dypsis lutescens* and *Trachycarpus fortunei*, preferentially sampled from indoor environments, such as buildings' lobbies, were possibly less exposed to fungal infections and, consequently, a smaller number of fungal isolates and genera were recorded from the leaf spotting communities of these hosts species.

Although the overall fungal assemblage pattern is similar in all host species, substantial differences in the assemblages of coelomycetes and hyphomycetes were found (Supplementary Figure A.3, Figure 3.2 C). Such as verified in the overall community, the percentage abundance of occurrence of hyphomycetes was higher than coelomycetes in all host species, except *Phoenix canariensis*, which presented a slightly higher percentage of coelomycete occurrences. *Dypsis lutescens* and *Trachycarpus fortunei* were the host species more enriched in hyphomycetous fungi. *Dypsis lutescens* was particularly impoverished in coelomycetous fungi.

The differences between hyphomycete and coelomycete assemblages in all host species can be seen not only in their percentage abundance of occurrence, but also in the composition of their main genera (Supplementary Figure A.3, Figure 3.2 C, Figure 3.3).

Alternaria was the most abundant hyphomycete genus in all host species, along with *Cladosporium* and *Epicoccum*. However, this trend was not followed in *Dypsis lutescens*, whose most important hyphomycete genera include *Penicillium* and *Pithomyces*, besides *Alternaria*. In *Chamaerops humilis* the third most important hyphomycete genus was *Stemphylium*. Besides these differences, in general, the hyphomycete

assemblage and the respective percentage abundance of occurrence for each main genus was relatively similar in all host species (Figure 3.3).

Phoma was the most abundant coelomycete genus in all host species, except in *Chamaerops humilis* where *Neosetophoma* was the most abundant. Compared to the hyphomycete assemblage, the differences in the main coelomycete genera between different host species were more relevant. Each host species, where percentage abundance of occurrence of coelomycete assemblage was relevant, was found to have a specific group of most abundant coelomycetous fungi in *Pleosporales*, besides *Phoma* (Figure 3.3).

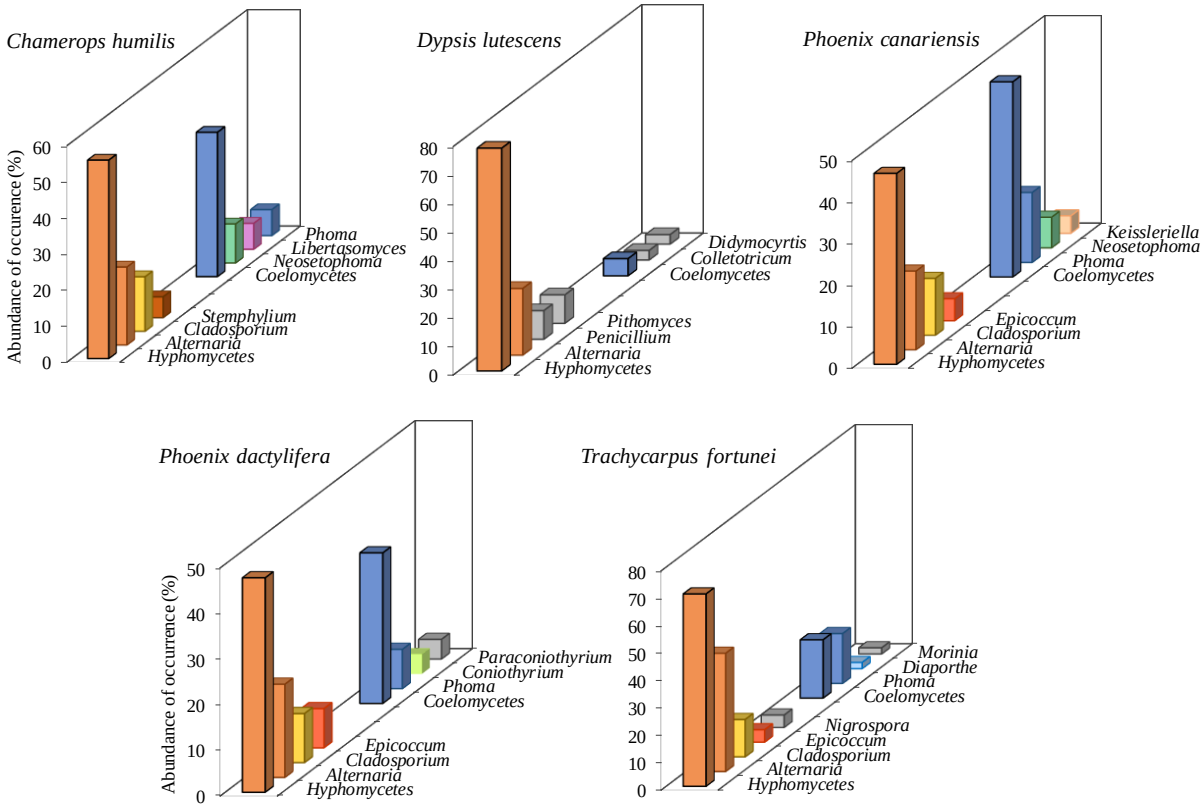

Figure 3.3 Main hyphomycete and coelomycete genera per host species. Percentage abundance of occurrence of hyphomycete and coelomycete assemblages and their corresponding three most important genera found associated with the foliar lesions of palms examined for each host species. Four genera are presented in *Trachycarpus fortunei* since the third and the fourth most important genera in each assemblage of this host species were reported with the same percentage abundance of occurrence. Colours are according to fungal types and fungal genera.

While hyphomycete assemblages are homogeneous in most of the hosts surveyed, coelomycete assemblages seem to be more heterogeneous (Figure 3.2 C, Figure 3.3). Thus, coelomycetes are the main cause of the differences detected in the mycota of different host species, since they include most of the rare and infrequent genera recorded. Although *Phoma* was the most frequent coelomycete genus for the overall fungal assemblage, contrary to *Alternaria* for the hyphomycetes, it is not the most frequently occurring genus in all the hosts surveyed. In *Chamaerops humilis*, *Neosetophoma* seems to be more frequently associated with the foliar lesions, while *Phoma* was recorded with the same percentage abundance of occurrence as *Libertasomyces*. *Libertasomyces* were only occasionally recorded in two other hosts and seems to be particularly abundant in *C. humilis*. It is interesting that these differences are occurring on the only palm host surveyed that display not only a temperate climate native range, but also a Mediterranean climate native range (Dransfield *et al.*, 2008; Gilman *et al.*, 2018), which is typical in Portugal. Somehow, mycota from *Chamaerops humilis* is displaying differences that could be related to the climatic constrains for this host in a similar extent to that differences reported in mycota of palms from tropical regions. It appears that a typical temperate palm mycota may exist that is expressed mainly in the coelomycete assemblage. This may justify why Taylor *et al.* (2000) reported that coelomycetes account for more of the fungi recorded from

palms in temperate regions, as well as why the pattern was also consistent with the present results. More intensive studies on wild representatives of *C. humilis* would probably clarify this pattern. Other evident differences on the main genera of coelomycetes found include *Neosetophoma* and *Keissleriella* recorded on *Phoenix canariensis* and *Coniothyrium* and *Paraconiothyrium* recorded on *Phoenix dactylifera*, which account for the high diversity of the fungal assemblage reported from these hosts.

Trachycarpus fortunei and, especially, *Dypsis lutescens* were shown to be particularly depauperate in the coelomycete assemblage. Sampling effort may be generating artificial differences, since *Dypsis lutescens* and *Trachycarpus fortunei* were the hosts with the lowest number of samples collected. In fact, considering that the coelomycete assemblage are mainly composed by rare and infrequent genera, its abundance and composition are highly affected by the sampling effort.

The differences between fungal assemblages recorded on different host species can also be quantified using biodiversity measures. Colonization rate, isolation rate, Simpson index of diversity, Shannon index of diversity and evenness index for the fungal assemblage associated with the foliar lesions of each host species are listed in Table 3.4. The ratio between the number of genera/number of samples (number of genera per sample) was also included, since genera is the principal component of the biodiversity analyses and ecological observations in this dissertation. This ratio can be used as a biodiversity measure, since it expresses how many different genera can be expected to be found per host species sample. Thus, this ratio is an indirect measure of the genera richness for a given host species and it is normalized with the number of samples examined, which can diminish sampling effort biases.

Table 3.4 Biodiversity measures of fungal assemblages per host species. Colonization rate (CR) and isolation rate (IR) were computed considering the number of samples examined from each host species and the correspondent number of isolates yielded. Number of genera per sample (foliar lesion) was calculated as the number genera recorded in each host species divided by the correspondent number of samples examined. Simpson index of diversity (D), Shannon index of diversity (H') and evenness index (J') were computed considering the number of genera and the number of isolates recorded for each host species. Host species with an absolute frequency of less than five samples examined were excluded due to sampling effort bias.

Host	CR	IR	Number of genera per sample	D	H'	J'
Chamaerops humilis	100%	6.86	1.14	0.90	1.14	0.83
Dypsis lutescens	100%	4.57	2.00	0.92	1.06	0.92
Phoenix canariensis	100%	3.77	1.00	0.91	1.17	0.83
Phoenix dactylifera	100%	8.00	2.25	0.92	1.22	0.85
Trachycarpus fortunei	100%	7.83	2.00	0.77	0.79	0.74

All the samples examined from each host species yielded at least one isolate. Thus, colonization rate was 100% in all host species. However, differences in isolation rate were found, as well as for the number of genera per sample (Table 3.4). Isolation rate is equivalent to the number of isolates yielded per sample, so it is an indirect measure of the degree of multiple colonization from the samples (Fröhlich *et al*., 2000a). *Dypsis lutescens* and *Phoenix canariensis* were two of the hosts whose samples presented the lowest isolation rate (4.6 and 3.8, respectively). A low isolation rate may indicate a low fungal richness. Thus, *Dypsis lutescens* and *Phoenix canariensis* were the host species with the lowest fungal richness per sample. Considering that from the set of the hosts examined, these present a tropical to subtropical native range (Dransfield *et al*., 2008; Broschat, 2017; Friedman *et al*., 2019a), growing them on a temperate climate may impoverish their mycobiota. As Taylor *et al*. (2000) showed with the studies on the tropical palm *Archontophoenix alexandrae*, tropical palms outside their natural habitat may be depauperate in palm fungi. The present results suggest that tropical and subtropical palms, such as *Dypsis lutescens* and *Phoenix canariensis*, introduced outside their natural range have a generally impoverished mycota. This phenomenon of depauperate fungal assemblages accruing on hosts growing outside of their native geographical range has been noted previously for pathogens of weed plants, such as *Chromolaena odorata* (Barreto and Evans, 1994), *Mikania micrantha* (Barreto and Evans, 1995) and *Lantana camara* (Barreto *et al*., 1995). Outside their native geographical range, these hosts showed assemblages composed of nonspecific, opportunistic pathogens. A similar result

can be found in the present study where the fungal assemblage recorded was mainly composed by widespread leaf spotting fungi.

A very different pattern is observed in typical temperate climate palm host species, such as *Chamaerops humilis* and *Trachycarpus fortunei*. Samples from these two palms showed a fungal richness per sample (7.8 and 6.9, respectively) almost twice as high as those from *Dypsis lutescens* and *Phoenix canariensis*, which is concomitant with the fact that these palms are adapted to temperate climate and, consequently, to its typical mycobiota. This may suggest that the low proportions of fungi associated with samples from *Dypsis lutescens* and *Phoenix canariensis* could be due to the lack of tropical palms in temperate regions which could "share" fungi with these hosts. It is important to highlight that the mechanisms behind the distribution of palm fungi are uncertain and only speculations can be made (Taylor *et al*., 2000). Not only were *Dypsis lutescens* and *Phoenix canariensis* impoverished in typical palm fungi, but these hosts were depauperate of fungi in general, since their foliar lesions were shown to yield a smaller number of plurivorous fungi per sample than the other palm host species examined, such as *Chamaerops humilis* and *Trachycarpus fortunei*, which are considered temperate palms (Dransfield *et al*., 2008; Gilman and Watson, 2014; Gilman *et al*., 2018). It seems clear that climatic requirements play a role in shaping fungal richness in different palm hosts species, although the pattern are not easy to clarify with the data obtained. A systematic survey of the mycota from wild stands of the temperate palm *Chamaerops humilis*, the only native palm in Portugal, would provide more definitive information to establish well-supported comparisons.

Phoenix dactylifera presented the highest isolation rate between all the host species analysed. In terms of climatic requirements, *P. dactylifera* is best adapted to dry and desert climates (Dransfield et al., 2008; Friedman et al., 2019b). Thus, compared with the remaining host species, it shows a native climate range between the wet tropical regions and the warm temperate ones. The high fungal richness recorded in this host could possibly be related to this intermediate climate context. Following the trends previously discussed, *P. dactylifera* might be able to "share" its mycobiota with temperate palms, which may be the reason why its fungal richness was comparatively greater than other palm host species with a native climate range different from the temperate climate, such as *D. lutescens* and *P. canariensis*, even if the number of samples collected was not very substantial. This pattern is also followed by the ratio number of genera/number of samples, since *P. dactylifera* was the palm host species with the highest value for this ratio. This indicates that it was not only the host with the highest number of isolates per sample, but also the host with the highest genera richness per sample. Some hypothesis here presented as trends could be tested with growth studies at different temperature regimes of isolates of palmicolous fungi, which would provide a direct method for testing some of their biological constrains.

The number of genera per sample is not easy to analyze, since it seems not to be directly related with the number of isolates. However, this could be biased by the percentage abundance of occurrence of each genus. If a given genus is very frequent on the samples of a certain host, it will decrease the corresponding number of genera per sample. Thus, while a high value of genera richness per sample could be directly related with biodiversity, a low value may only reveal the presence of very frequent genera in a great number of samples from that host. As can be seen in Table 3.4, the number of genera per sample do not vary enough to explain patterns of biodiversity, since it ranges from one to just over two genera per sample. Additionally, it seems that no association exists between the host native climate range and the number of genera per sample.

Analysis of fungal biodiversity in different hosts was also carried out using diversity indices, such as the Simpson index of diversity and the Shannon index of diversity, as well as the evenness index. Differently from genera richness per sample, that only considers the number of genera recorded, diversity indices combine genera richness and abundance into a single value. Although more informative, this also makes interpretation difficult as diversity indices with the same value may arise from various combinations of genera richness and abundance. Thus, values for diversity indices presented in Table 3.4 should be interpreted with care.

To aid interpretation, a measure of abundance was estimated using an evenness index based on the Shannon index of diversity. The closer the value is to one, the more evenly spread the individuals are between the different genera recorded and, consequently, more diverse is the fungal community. Apparently, in all

the hosts analyzed, the fungal communities recorded showed a similar biodiversity. However, it is interesting that *Dypsis lutescens* seems to be relatively more diverse, since its evenness index was higher than the other hosts. Although impoverished in fungi, *Dypsis lutescens* maintains a fungal community with a relatively high diversity. This is directly related with the fact that this palm host is particularly depauperate in coelomycetes and all the coelomycetes recorded are represented by a very low number of individuals, consequently increasing the evenness of the fungal community.

Another interesting case is the lower values for diversity indices recorded in *Trachycarpus fortunei*. In this case, the diversity is particularly affected by the high number of isolates of *Alternaria* recorded. The dominance of this genus not only decreases the values for the diversity indices, but also decreases the equitableness of the fungal community, which is represented in 50% of all records by *Alternaria* isolates.

It is not surprising that the diversity indices mostly revealed similar levels of the diversity of the fungal communities surveyed for each host.

First, as noted, all the plants surveyed are ornamentals and are subjected to the same climate constrains, the warm temperate Portuguese climate. Secondly, the distribution of the genera is very alike in all hosts surveyed (Supplementary Figure A.3). Assessing the absolute frequency for each genus occurrences, it is possible to verify that, although different genera compose each host fungal assemblage, the pattern is very alike in all of them: few dominant genera and a wealth of rare genera that contributes for the homogeneity of the community, especially in the coelomycete assemblage. So, differences in the fungal assemblage of each host are particularly related with the presence of different rare genera, some of them only appearing in a specific host (exclusive genera) and thus contributing to increase the diversity of the fungal assemblage recorded in that host. The number of exclusive genera was higher in *Phoenix dactylifera* and lower in *Trachycarpus fortunei* (Figure 3.2 D), which is concomitant with the previous discussed results.

3.3. Fungal assemblages and parishes: are different parishes harbouring different fungal genera communities?

Different sampling sites were assessed to search for foliar lesions on ornamental palm trees, including Oeiras and five Lisbon parishes, namely Alvalade, Areeiro, Marvila, Parque das Nações and São Vicente. A different number of samples was collected in each site (Figure 2.1, Figure 3.4 A). While in Oeiras and São Vicente only two and three foliar lesions were collected, respectively, a greater number of samples was collected in the remaining parishes, including seven in Areeiro, 17 in Alvalade, 20 in Marvila and 29 in Parque das Nações. Oeiras and São Vicente were excluded from the biodiversity analyses, since the number of trees examined and, consequently, the number of foliar lesions collected (less than five) were too small to support any trend observations. Note that a great diversity of foliar lesions types and palm tree species were collected and examined in each parish, except for Areeiro, where most of the trees examined were *Phoenix canariensis* (Supplementary Table A.4, Supplementary Table A.5).

Following the trends already observed at the palm host species level, in general, the greater the number of samples collected, the greater the genera and fungal richness recorded. Thus, Parque das Nações was the parish with the highest number of genera and isolates recorded. Although the number of samples collected in Marvila was slightly higher than the number of samples collected in Alvalade, the number of isolates recorded was similar in both parishes and the number of genera was higher in Alvalade. Areeiro showed a considerably small number of isolates and genera, concomitant with the low number of samples collected in this parish. Nevertheless, to include the results obtained in Areeiro in the present analysis is important, since it can reveal some biases from the sampling effort (Figure 3.4 A).

In each parish the number of samples that yielded more than two isolates were considerably higher than the number of samples that yielded both one and two isolates. Nevertheless, proportionally, Parque das Nações showed a greater number of samples with multiple fungal colonization, which is concomitant with the isolation rate computed for samples from this parish (Figure 3.4 B, Table 3.5).

Differences in hyphomycete and coelomycete assemblages can be found in each parish (Supplementary Figure A.5). While Marvila was the parish with the lowest relative percentage of coelomycetes (just over 20%), Areeiro was the Parish that showed the highest relative percentage of coelomycetes (more than 40%).

Parque das Nações and Areeiro presented a similar percentage of coelomycetes and hyphomycetes, contrary to Marvila and Alvalade, where hyphomycetes seem to be the main fungal assemblage (Figure 3.4 C).

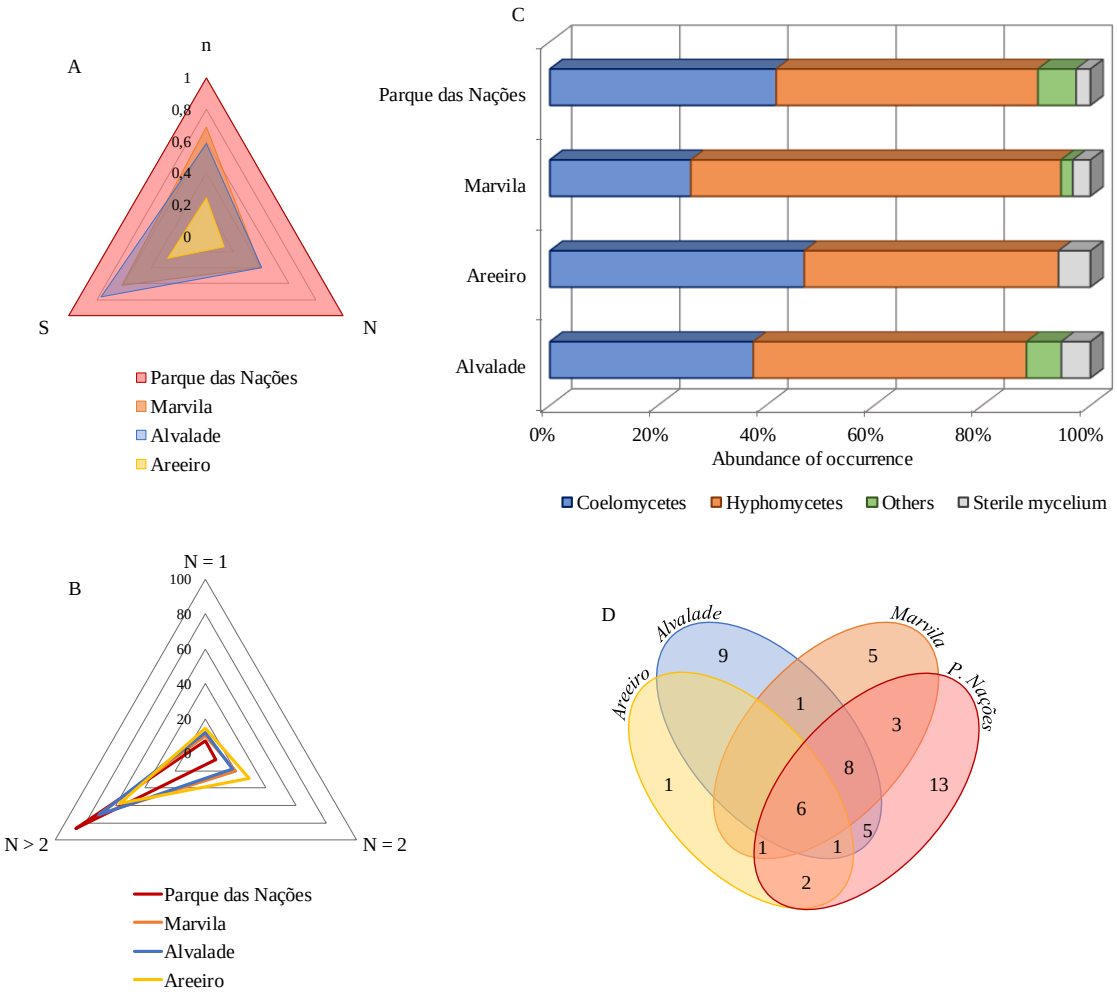

Figure 3.4 General biodiversity patterns of fungal records per parish. **A.** Graphical representation of the relative frequency of isolates and genera yielded, and foliar lesions collected for each parish. Each variable was normalized by its maximum value, so all three variables could be plotted together, otherwise differences in scale values between the number of isolates and the remaining variables would impair data visualization. **B**. Graphical representation of the number (%) of samples that yielded 1 isolate (N = 1), the number of samples that yielded 2 isolates (N = 2) and the number of samples that yielded more than 2 isolates (N > 2) for each parish. **C**. Percentage abundance of occurrence of each fungal type for each parish. **D**. Venn diagram of genera richness by parish. Parishes with an absolute frequency of less than five foliar lesions examined were excluded due to sampling effort bias. In A, B and D colours are according to parishes. In C colours are according to fungal types. n = number of samples, N = number of isolates, S = genera richness.

A different perspective arises when evaluating the number of genera of each assemblage per parish. In Parque das Nações the genera richness of coelomycetes was almost three times higher than that of hyphomycetes, contrary to what was recorded in the remaining parishes, where the genera richness of both assemblages was quite similar (Figure 3.5 A). Additionally, as can be seen in Figure 3.5 B, the coelomycete assemblage was more even in their genera abundances of occurrence than hyphomycetes assemblage, where *Alternaria* dominance was always remarkable. Nevertheless, this genus was particularly dominant on Marvila, where it composes more than 30% of the fungal community. In Areeiro, both assemblages were quite even and *Alternaria* co-dominate the hyphomycete community with *Cladosporium* and *Stemphylium*. This co-dominance, with the addition of *Epicoccum*, was also observed in Parque das Nações, although not so evident due to the increase in *Alternaria* records. The number of residual genera was higher in Parque das Nações and Alvalade (Figure 3.5 A, Supplementary Figure A.5).

Concomitant with the previous results, Parque das Nações was the parish with the highest number of exclusive genera (13 of all genera records), followed by Alvalade (9) and Marvila (5). The number of genera

shared between Parque das Nações and Marvila or Alvalade was quite similar and higher than the number of genera shared between Marvila and Alvalade (Figure 3.4 D).

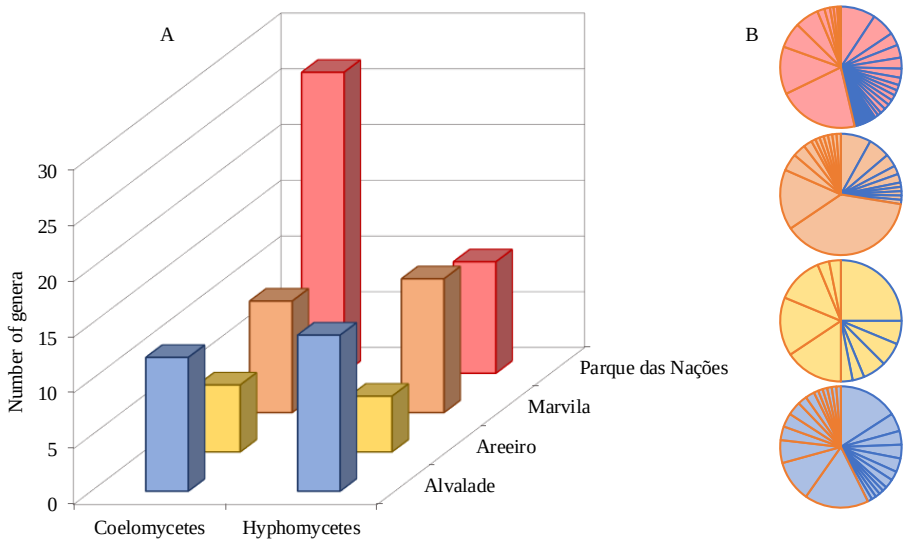

Figure 3.5 Genera richness and abundance of hyphomycete and coelomycete assemblages per parish. **A**. Number of genera of coelomycetes and hyphomycetes found associated with the foliar lesions collected from each parish. Colours are according to parishes. **B**. Pie-charts representing the percentage abundance of occurrence of each fungal genus found associated with the foliar lesions collected from each parish. Slices' fill colours are according to parishes, where slices' line colours are according to fungal types, where blue represents coelomycetes genera and orange represents hyphomycetes genera.

All these patterns can be evaluated numerically with the diversity indices computed and are presented in Table 3.5. All the samples examined from each parish yielded at least one isolate. Thus, colonization rate was 100% in all parishes. However, differences in isolation rate were found. Parque das Nações was the parish with the highest isolation rate, followed by Alvalade, Marvila and Areeiro. A different result was found for the number of genera per sample. Although Alvalade presented a slightly higher genera richness per sample, it was very similar in all parishes (Table 3.5).

Table 3.5 Biodiversity measures of fungal assemblages per parish. Colonization rate (CR) and isolation rate (IR) were computed considering the number of samples examined in each parish and the correspondent number of isolates yielded. Number of genera per sample (foliar lesion) was calculated as the number of genera recorded in each parish divided by the correspondent number of samples examined. Simpson index of diversity (D), Shannon index of diversity (H'), evenness index (J') and Margalef species richness index (D_{Mg}) were computed considering the number of genera and the number of isolates recorded in each parish. Chao1 species richness estimator (S_{Chao1}) was computed considering the number of singletons and doubletons genera recorded in each parish, along with the genera richness. Parishes with an absolute frequency of less than five samples examined were excluded due to sampling effort bias.

Parish	CR	IR	Number of genera per sample	D_{Mg}	S_{Chao1}	D	H'	J'
Alvalade	100%	5.47	1.76	6.40	54.50	0.94	1.29	0.87
Areeiro	100%	4.29	1.60	2.94	13.67	0.89	0.93	0.89
Marvila	100%	4.60	1.20	5.09	56.67	0.83	1.02	0.74
Parque das Nações	100%	8.00	1.34	7.00	63.50	0.92	1.30	0.82

Margalef species richness index and Chao1 species richness estimator were as high or higher in Parque das Nações and Alvalade compared to the remaining parishes, which seems to predict that these parishes presented a higher potential fungal community diversity. This was concomitant with the values computed for the diversity indices, which indicate that, in general, the diversity and equitableness of Alvalade and Parque das Nações fungal communities were higher. The evenness index for Marvila fungal community was lower compared to Areeiro, pointing to its decreased diversity. Shannon index of diversity differences between parishes were higher than those presented by Simpson index of diversity, showing clearly that fungal

diversity in Parque as Nações and Alvalade was higher than the fungal diversity found in Marvila and Areeiro (Table 3.5).

The overall results obtained may indicate that Alvalade and Parque das Nações were the sampling sites with the highest fungal community diversity. Although these results may be, in part, biased due to sampling effort, trends can be pointed out when the variables are compared considering the number of samples examined. The number of isolates and the genera richness were considerably higher in Parque das Nações compared to the other parishes (Figure 3.4 A). This was not only a bias product of the sampling effort, since isolation rate and Margalef index also show the highest value in the fungal community from Parque das Nações (Table 3.5). Margalef species index richness attempts to compensate for sampling effects by dividing richness by the total number of individuals recorded (Mangurran, 2004). Thus, although still strongly influenced by sampling effort, Margalef index seems to be a more reasonable measure for genera richness in the present study, and Parque das Nações is here reported as the parish which fungal community present the highest genera richness. This is concomitant with the isolation rate recorded for this parish, which was almost 4 times higher than the isolation rate recorded for the remaining parishes. Note that, isolation rate can be used as a measure of the fungal richness, thus Parque das Nações was the collecting site that showed not only the highest genera richness, but also the highest fungal richness. Alvalade and Marvila also presented a considerably high value for genera richness, which were similar in both parishes (Figure 3.4 A). Nevertheless, Margalef index was higher in Alvalade fungal community than in Marvila, with a value close to that one computed for Parque das Nações (Table 3.5). In this sense, taking into account that the number of samples collected in Alvalade was smaller than in Parque das Nações, Alvalade seems to present a genera richness as high or higher as that in Parque das Nações. In fact, the number of genera recorded per sample was slightly higher in Alvalade than in Parque das Nações (Table 3.5). Areeiro was included in this analysis to unveil the possible bias associated with the sampling effort, since the number of samples collected in this parish was seven, which is only two samples above the limit below which the parishes where excluded from the analysis. This bias could be seen, for example, comparing the number of genera per sample and the Margalef index obtain to this parish. While the Margalef index reveals a very low genera richness, as expected considering that the number of samples examined was too small, the number of genera per sample greatly increases for the same reason. This kind of bias should, however, be decreased for the remaining parishes, where a considerable number of samples have been examined assuaging these effects.

Parque das Nações lies on a strip of land 5 km long by the river Tejo and a third part of which is made up of green spaces (Portal das Nações, 2014). All the sampling sites in Parque das Nações were located near the river and all these sites had a high concentration of trees of different species. This humid location by the Tejo estuary, along with the high concentration of trees, may be playing a decisive role in the diversity of the fungal communities due to the generation of an optimal microclimate for fungal growth, sporulation, dispersal, germination and infection (Agrios, 2005). This could be one of the reasons why palm foliar lesions from Parque das Nações showed a several times higher isolation rate than the other parishes (Table 3.5), which is concomitant with the high number of foliar lesions that yielded more than 2 isolates (Figure 3.4 B). The influence of relative humidity due to evaporation (Zhu *et al*., 2017) on the sporulation of leaf spotting fungi and the expansion of leaf spot lesions were reported several times in the literature (Jhorara *et al*., 1998; Talley *et al*., 2002; Paul and Munkvold, 2005; Shrestha *et al*., 2010; Chowdhury and Hossain, 2011; Selvamani *et al*., 2014; Rowlandson *et al*., 2015). In addition, the leaf spotting fungal community recorded from this parish presented a higher diversity in different aspects, which comprises from the genera richness to the fungal richness and the number of exclusive genera (Figure 3.4 A, B and D).

The amount of diseased foliar tissue observed in Parque das Nações seems to be another factor supporting the previous hypothesis, since it was clearly greater than in the remaining parishes. In fact, it has been already reported that the number of leaf spots observed on palms in nurseries tend to be much greater than in the forests due to the crowded nursery conditions (Fröhlich *et al*., 1997). Palms in Parque das Nações were always found in crowded green spaces, with several different plant species, to create an exotic landscape architecture. The proximity between different trees may have facilitated the flow of fungal spores, which combined with the moisture conditions may have potentiated a greater development of foliar lesions.

Although the previous analysis has been done to Parque das Nações, a similar effect is expected to be occurring in Alvalade. In fact, the fungal diversity recorded in Alvalade was also remarkably high and in some biodiversity measures very similar to that recorded in Parque das Nações (Table 3.5). Most of the sampling sites in Alvalade also included several palms near the lake of Jardim Mário Soares. Thus, the effect of increased humidity due to evaporation (Zhu *et al*., 2017) may also be playing a decisive role in the high diversity of the fungal community recorded in Alvalade.

Parque das Nações was the only parish where the genera richness of coelomycete assemblage greatly surpasses the genera richness of hyphomycetes assemblage (Figure 3.6 A). As previously discussed, coelomycetes assemblage is possible the main composition of the palm fungal mycobiota in temperate regions and in the present study is the main assemblage contributing to the great biodiversity recorded. Most of the rare and infrequent genera, that greatly increase the biodiversity of the fungal community, are present in the coelomycete assemblage and almost all the coelomycete genera recorded in the present study are represented in the coelomycete assemblage from the foliar lesions collected from Parque das Nações (more than 81% of all coelomycete genera records) (Supplementary Figure A.5). This is one of the main differences between the diversity recorded in Alvalade and in Parque das Nações, since most of the hyphomycete genera recorded in the present study were found in Alvalade, thus the diversity of Alvalade's fungal community may be mainly related with its hyphomycete assemblage (Supplementary Figure A.5). While in all the other parishes both assemblages are quite even in their genera richness, the geographical conditions previously described seem to potentiate the development of a more diverse and coelomycetous fungal community in Parque das Nações, although the percentage abundance of occurrence of Hyphomycetes was higher in all parishes (Figure 3.5).

The fungal community in Parque das Nações seem to be more even in their genera distributions (Figure 3.5 B), although this is not expressed by the evenness index computed probably due to the abundance of *Alternaria* isolates (Table 3.5). It is interesting to note that the number of different foliar lesions types collected in this parish was quite similar and this is probably another reason why the fungal community was shown to be so diverse and even (Supplementary Table A.5). In fact, while the coelomycetes assemblage are coming mostly from tip die-back diseases and large leaf spots (44% of all the foliar lesions collected from Parque das Nações), the hyphomycetes assemblage are coming mostly from small leaf spots and pinpoints and punctuations (66%), which justifies the high coelomycetous genera richness, as well as the high abundance of hyphomycetous records. A similar result was observed in Alvalade. However, the percentage abundance of occurrence of *Phoma* was much higher than that recorded in Parque das Nações (Figure 3.5 B), what consequently decreases the evenness of the coelomycetes assemblage and the diversity in general. It is interesting to note that in Parque das Nações, the hyphomycetes assemblage shows a co-dominance pattern of four different genera – *Alternaria*, *Cladosporium*, *Stemphylium* and *Epicoccum* – contrary to what is observed in the remaining parishes, where *Alternaria* isolates clearly dominates the fungal assemblage (Figure 3.5 B). This is another aspect that unveils the great diversity of the fungal assemblage in the foliar lesions collected from this parish. A similar co-dominance pattern, excepted for the absence of *Epicoccum*, was observed in Areeiro, but the number of foliar lesions examined was too small to consider it a pattern of biodiversity (Figure 3.4 A, Figure 3.5 B).

Evaluating the sampling process: how far from the truth?

In order to evaluate whether enough samples were taken at each sampling site, it was necessary to examine the relationship between increasing the sample size and recovery of genera. Thus, a genera-accumulation curve was generated for each parish and for the overall fungal community surveyed from all the foliar lesions examined (Supplementary Figure A.4). In each case, the genera-accumulation curve did not reach an asymptote, insomuch that the slopes of the curves were only declining momentarily with the increasing number of samples. Thus, it was not possible to predict the number of samples at which the slopes would become near zero, which would predict the expected number of genera possible to find at each parish or for the overall fungal community.

The overall number of genera recorded in each sampling site was different, which suggests that their genera richness may be also different (Table 3.5). Nevertheless, genera-accumulation curve can also help to unveil these biodiversity patterns by evaluating the steep gradient of each curve. The steep gradient for the genera-accumulation curve of Alvalade is higher than that for Parque das Nações and Marvila, both with a very similar slope, although different sampling effort should be taking into consideration.

Due to possible sampling bias, no best curve-fitting model was tested to predict the inflection point of each curve. Nevertheless, considering that their steep gradients never expressively level off, each curve was tested for linearity and slope significance of the regression (Zar, 2010). Given that the values obtained for the determination coefficient (R^2) were above 0.97, the calibration curves for the genera-accumulation curves for Alvalade, Marvila and Parque das Nações were accepted. This revealed that the sampling process was still in a constant rate increasing phase in terms of genera richness and far from attaining an eventual plateau. The sampling effort for Areeiro was too low to predict its genera-accumulation curve, although it is presented along with the others, but the R^2 of its calibration curve was too low to be accepted and nothing should be concluded using these data (Supplementary Figure A.4).

Evaluating the adequacy of sampling size is highly important on the present study, considering that all the biodiversity patterns and ecological observations here presented may be a product of biased sampling. Most of the collections were made randomly both in terms of collection sites and host species examined. Although ornamental palms are broadly present throughout Portuguese cities, their canopies are not always accessible to search for the presence of foliar lesions due to the height of the trees. None of the curves had levelled-off, which indicates that many fungal representatives remain to be isolated and identified.

Besides the fact that genera-accumulation curve can be used to indicate the adequacy of the fungal survey, it can also be used to estimate the number of genera in a particular area. However, a best curve-fitting model would be necessary to predict the asymptote for each curve, since this value would be equal to the number of genera present in the community (genera richness) (Thompson and Withers, 2003; Thompson *et al*., 2003). Nevertheless, the diversity of the fungal communities of each parish can be compared also with the genera-accumulation curve. It is known that sites with high diversity have steeper initial slopes for their collector's effort curves (Thompson and Withers, 2003). The genera-accumulation curve for Alvalade has a steeper slope gradient then that for Parque das Nações and Marvila. In this sense, contrary to the previous analysis, Alvalade seems to have a more diverse fungal community and it is expected to potentially contain a higher plethora of unrecorded genera.

Since no best curve-fitting model was tested, Chao1 species richness estimator was used to estimate the richness of each parish and to predict how far from the true value of the genera richness is the present survey. Chao1 is a nonparametric method that calculates the expected genera richness value based on the observed genera richness as a measure of estimating the number of genera in a community (Chao, 1984). This estimator is based on the concept that rare genera infer the most information about the number of missing genera (Kim *et al*., 2017). Thus, it seems reasonable to use this estimator to predict the number of genera that were not recorded in the present study, since it is particularly useful for data sets skewed towards the low-abundance species (Hughes *et al*., 2001). The values obtained for Chao1 species richness estimator was 55 for Alvalade, 57 for Marvila and 64 for Parque das Nações. The value obtained for Chao1 species richness estimator was 97 for the overall community, i.e., 97 genera are expected to exist in the leaf spotting fungal community from the foliar lesions of palms in Portugal. Considering that only 57 genera were recorded in the present study, this means that only just over 50% of the potential leaf spotting mycota was recorded. Although this study revealed a plethora of fungal diversity capable of unveiling certain ecological traits, it is far from the true diversity that exists in the foliar lesions from palms.

It is likely that many fungal taxa were present on the foliar lesions surveyed but were not isolated, such as: (a) obligate biotrophs; (b) fungi that are difficult to isolate without the use of selective media; (c) fungi that were possibly killed during the surface sterilization protocol; (d) seasonal transients, i.e., fungi that are present in the leaf spotting fungal communities at times other than those of sampling (Fröhlich and Hyde, 1999).

3.4. Foliar lesions types: an artificial concept or a new way of unveiling fungal communities' ecology?

A well-defined sample unit is of utmost importance in biodiversity studies (Zak and Willig, 2004), since it will ensure the diminishing of possible bias that could compromise the correct interpretation and establishment of ecological patterns and trends. The sample unit in the present study corresponds to the foliar lesion, which has been defined as a set of discretely localized spots on the host leaves. In this sense, several morphological characteristics were assessed to characterize each foliar lesion and a randomly selected representative spot was chosen to study its fungal community.

A total of 78 foliar lesions were collected and examined for associated fungi. Four different foliar lesions types – tip die-back, large leaf spots, small leaf spots, and pinpoints and punctuations – were defined according to their general abaxial and adaxial surfaces morphological characteristics (Figure 3.6), namely shape, margin topography, colour and its evolution over time (when possible), presence or absence of a distinctive border, halo or occasional coalescence, overall distribution on the leaflet, and the size (if applicable).

Tip die-back (TDB; Figure 3.6 A,a,b) foliar lesions were extensive necrosis on leaflets or leaf segments beginning at their tips and advancing toward their bases. Morphologically they were irregular, with rounded ends, with different length depending on the lesion age, identical on both surfaces, occasionally paler on the abaxial surface, pale-grey, pale-brown to brownish centre, usually becoming greyish and fragile or brittle, often with a dark-brown border (< 1 to 3 mm wide, rarely < 5 mm wide), rarely surrounded by an inconspicuous to visible yellowish, light-brown to light-green halo, distributed on the edge of the leaflet. Usually in mature lesions contained several immersed to subimmersed or erumpent fruiting bodies, found on both surfaces, usually in a lower number on the abaxial surface, or only on the adaxial surface.

Large leaf spots (LLS; Figure 3.6 B,c,d) were extensive blotches, rarely with necrotic tissue, that were not exclusively associated with foliar tips. Morphologically they were ellipsoidal to irregular, > 10 cm in length, often bigger due to coalescence, with rounded to angular ends, identical on both surfaces, occasionally paler on the abaxial surface, pale-brown to brown, yellowish to greyish centre with dark-brown border (< 1 mm to 2 mm wide, occasionally thicker, darker and up to 10 mm wide), rarely becoming fragile or brittle, occasionally surrounded by an inconspicuous pale-whitish, yellowish to brownish halo, frequently with conspicuous concentric growth lines, often acquiring the appearance of overlapping layers, randomly distributed on the leaflet. Rarely in mature lesions contained immersed to subimmersed fruiting bodies, which often developed on sunken tissue regions.

Small leaf spots (SLS; Figure 3.6 C,e,f) were small discrete lesions on the foliar tissue. Morphologically they were subglobose to broadly ellipsoidal or fusiform, often becoming irregular, 1 to 5 cm in length, often larger, with rounded to angular ends, identical on both surfaces, occasionally paler on the abaxial surface, brown-grey, pale-brown, yellowish to greyish, sometimes blackish, centre with dark-brown border (up to 1 mm wide), rarely becoming brittle on the abaxial surface, surrounded by a conspicuous, rarely inconspicuous, paler, brown to yellowish halo, occasionally coalescing, randomly distributed on the leaflet, rarely found along the main vain. Rarely in mature lesions contained several immersed to subimmersed fruiting bodies, more often found only on the adaxial surface, or several conidiophores associated with stomatal apertures on the abaxial surface.

Pinpoints and punctuations (PP; Figure 3.6 D,g,h) were very small discrete lesions dispersed along the foliar tissue. Morphologically they were oval, globose to subglobose, ellipsoidal to fusiform, often irregular, < 1 cm in length, rarely up to 1.5 cm, with rounded to angular ends, identical on both surfaces, pale to yellowish, brown to blackish, usually surrounded by a conspicuous to inconspicuous brownish, yellowish to light-green halo, rarely absent, occasionally coalescing, randomly distributed along the leaflet, rarely found along the main vain or along the leaflet margins.

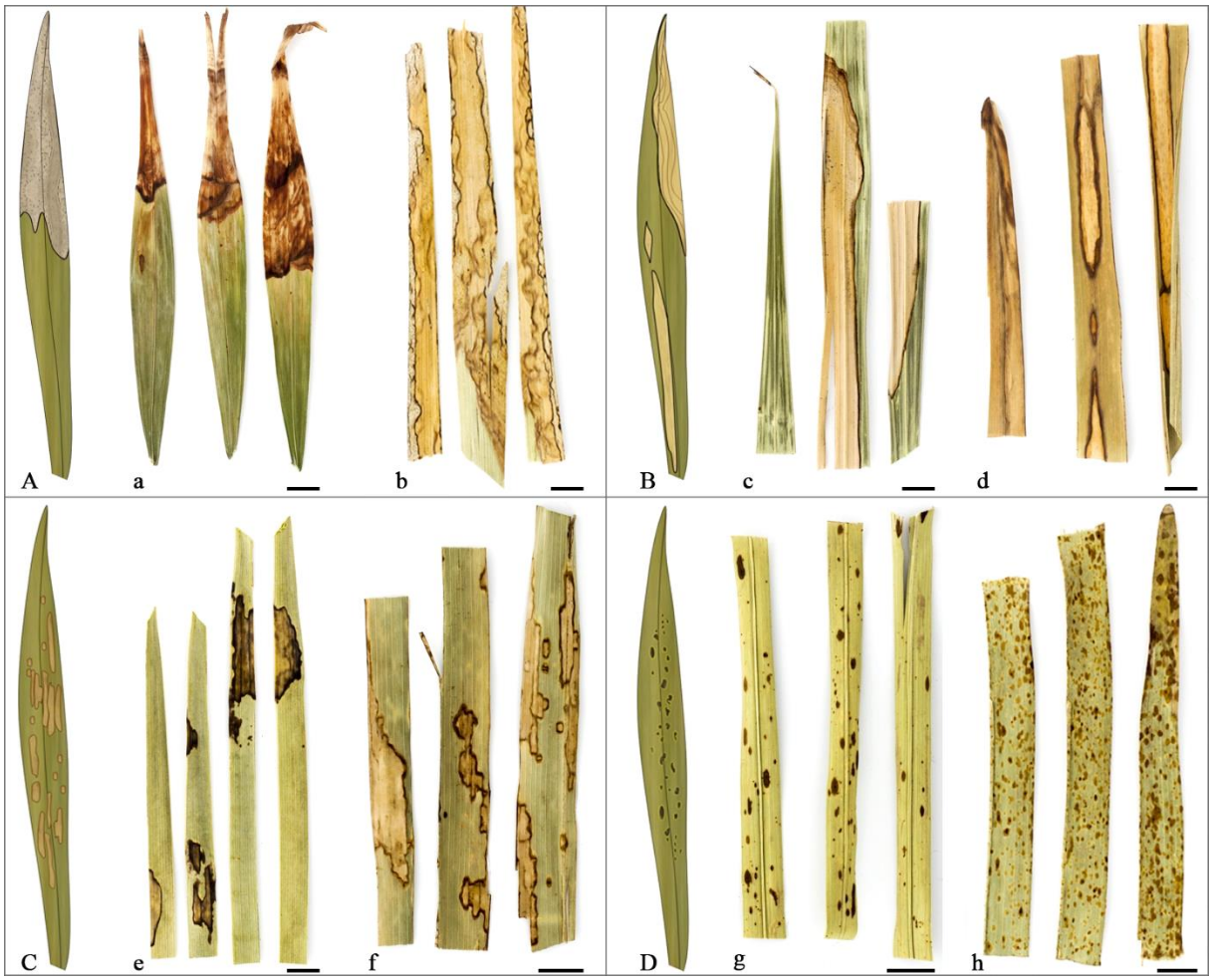

Figure 3.6 General morphology of foliar lesions types. **A**. Illustrative scheme of TDB lesions morphology, **a**. Sample HDP 006, **b**. Sample HDP 041. **B**. Illustrative scheme of LLS lesions morphology, **c**. Sample HDP 009, **d**. Sample HDP 042. **C**. Illustrative scheme of SLS lesions morphology, **e**. Sample HDP 033/02, **f**. Sample HDP 050. **D**. Illustrative scheme of PP lesions morphology, **g**. Sample HDP 004/01, **h**. Sample HDP 049.

Although the foliar lesions types were defined as "morphogroups", analyses of their associated mycota seems to indicate that these groups are not an artificially concept. In fact, it is known that several traits observed as symptoms in the foliar lesions are a consequence of the fungal mycota damaging the plant tissue, the defence mechanisms mediated by the plant and the interaction between these two components (Agrios, 2005). Thus, different morphological characteristics observed in the foliar lesions of palms can be attributed to the fungal growth and the subsequent plant response mechanisms. Three relevant examples, namely the size, the halo and the shape, are discussed below.

Foliar lesions size was one of the main traits use to defined types. While TDB and LLS were large, usually unmeasurable and occupying entire leaflets or leaf segments, SLS and PP were smaller, attaining a more or less specific and measurable size. Foliar lesions sizes may be dependent on the host and the fungal assemblage with them associated. For example, leaf spots caused by *Septoria* species vary widely in size from barely visible to spots that affect up to one-third of the leaf area, depending on the host and fungus species (Agrios, 2005). Thus, foliar lesions size is an essential trait to characterize, since it may be associated with the composition of the fungal community that is inhabiting the damaged plant tissue. Differences in the fungal community are expected to reflect differences in this morphological trait. In addition, if in the fungal community there are elements capable of infecting large areas of leaf tissue, this may influence their entire composition, since a greater amount of dead tissue and nutrients may become accessible to other fungal elements. In fact, considering that a larger foliar lesion damage encompasses a larger amount of nutrients available, this may strongly affect the respective fungal community. Nutrient acquisition is one of the key factors that determine the success of colonization during a parasitism lifestyle. Besides this, the pathogen's

fitness is also constrained by the plant defences, which contradicts the fungal growth and spread (Hedge and Fluhr, 2007; Fernandez *et al*., 2014; Maupetit *et al*., 2018). Thus, considering that foliar lesions correspond to localized lesions on the host leaves consisting of dead and collapsed cells, the size of these lesions may influence or be related to the fungal community lying there and the plant response to its spread.

In several foliar lesions, particularly in PP type, a more or less conspicuous yellowish to light-green halo surrounding the damaged tissue was observed. These halos represent tissue chlorosis and are formed due to chlorophyll destruction or failure of chlorophyll formation. Different leaf spotting fungi are known for producing these chlorotic halos as a consequence of toxin production (Agrios, 2005). For example, leaf spots caused by *Alternaria* spp. are often associated with the development of a chlorotic halo surrounding the necrotic tissue due to the production of toxins (Dewdney, 2013; Meena and Samal, 2019). Given that, the presence or absence of a chlorotic halo is expected to be associated with the fungal community on the foliar lesions examined. Differences in that fungal community are in turn expect to reflect differences in this morphological trait. Only PP were consistently associated with chlorotic halos surrounded the dead plant tissue, which can indicate that these foliar lesions were often associated with fungal elements capable of producing toxins. Whether these toxins are being produced by one or more fungal elements that compose the fungal community (which may or not have been isolated) would only be possible to determine through pathogenicity tests.

The shape of the foliar lesions was often less informative than the other morphological characters, since in general all foliar lesions showed similar globose to ellipsoidal shapes. Nevertheless, very few cases presented consistently a much greater length than width, acquiring fusiform shapes. Other cases presented angular, instead of rounded, ends. According to Agrios (2005), xylem vessels block the spread of some fungal pathogens, giving rise to various angular-shaped leaf spots due to their spread only into areas between veins. In the present study, fusiform-shaped leaf spots were particularly found localized near the main vein, possibly as a result of the plant defense mechanisms. Thus, the plant response to the pathogen growing will determine the shape of the lesion, which will subsequently spread in different directions avoiding the defence mechanisms. Given that, foliar lesions shape is clearly a consequence of the fungal colonization and the plant response in preventing its spread. Differences in that fungal community may reflect differences in this morphological trait, although in general shape has shown to be a more or less constant morphological character.

These three examples show that foliar lesions types may function as "morphogroups" of different fungal communities and its applicability may not be artificial. Foliar lesions are being interpreted as fungal communities, i.e. a set of fungi from one or more genera, interacting with the plant tissue. Thus, the symptoms and signs observed are expected to arise directly from this host-fungus interaction. In fact, how quickly the plant recognizes the pathogen and mobilize its defences determines how rapidly the infection will spread and establish. Subsequently, this determines how much the pathogens will develop and how severe will be its symptoms (Agrios, 2005). The observed morphological characteristics, namely the size, the halo and the shape, are then considered the expression of this interaction. It is important to note that these "morphogroups" did not take into consideration the host where the fungal community was thriving. A different and more complete perspective may probably arise if the palm species have been added to the equation. For example, *Dypsis lutescens* leaves were depauperated in coelomycetes probably because its leaves are softer and thinner compared to the other hosts surveyed. Thus, differences in foliar lesion morphology may arise from different palm hosts, although the present study did not consider their influence since no standardized sampling process was followed.

Is there any association between lesion types and their mycobiota?

In general, a similar number of all the foliar lesions types were found in each host species and parish, except for some particular cases (Supplementary Table A.3, Supplementary Table A.4). Yellow faded or translucent areas were frequently detected on leaves of *Chamaerops humilis*, but numerous attempts failed to isolate any possible fungal cause. These lesions were excluded from the foliar lesions set, firstly because they were associated with only one of the hosts surveyed, and second because they were not discrete spots

on the leaves, but rather faded areas that were difficult to define and not fitting within the initial defined concept.

Differences in the fungal communities isolated from each foliar lesion type were found and are summarized in Figure 3.7. A similar number of the different foliar lesion types were collected, including 19 TDB, 18 LLS, 17 SLS and 24 PP. While the number of genera found in each foliar lesion type was similar, the number of isolates varied substantially in part due to the collection effort, since a higher number of isolates were obtained from PP (Figure 3.7 A). Nevertheless, assessing the number of isolates (fungal richness) per sample, it is possible to verify that TDB lesions yielded the greatest number of isolates per sample, followed by PP. The number of genera (genera richness) per sample was similar in all four types of foliar lesion (Figure 3.7 C).

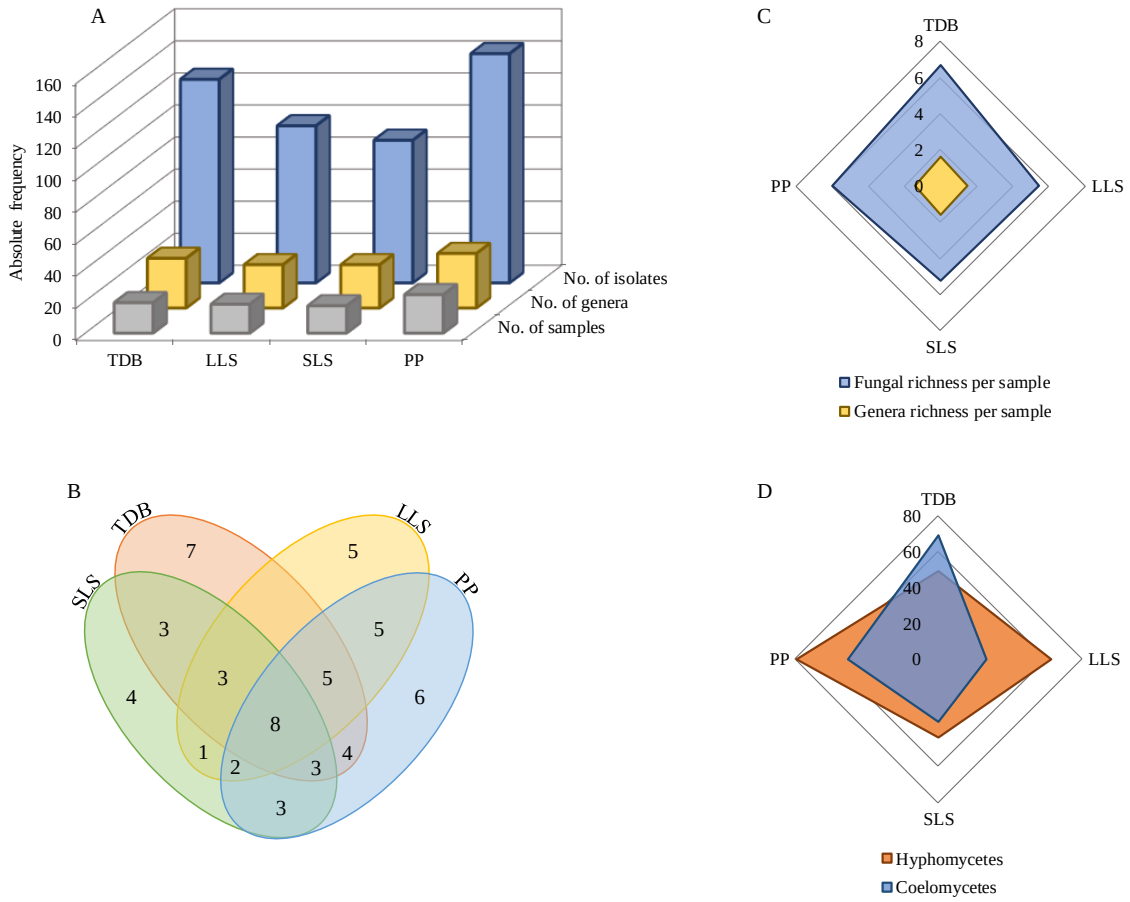

Figure 3.7 General biodiversity patterns of fungal records per foliar lesion type. **A.** Number of isolates and number of genera yielded, along with the number of samples examined per each foliar lesion type. **B.** Venn diagram of genera richness by foliar lesion type. **C.** Graphical representation of fungal richness and genera richness yielded per sample. **D.** Graphical representation of the absolute frequency of hyphomycetes and coelomycetes isolated from each foliar lesion type. In B colours are according to foliar lesion types. In D colours are according to fungal types. TDB = tip die-back, LLS = large leaf spot, SLS = small leaf spot, PP = pinpoints and punctuations.

The main difference between the fungal communities isolated from each foliar lesion type lies in the coelomycete and hyphomycete assemblages. While TDB lesions yielded mainly coelomycete isolates, LLS and PP yielded mainly hyphomycete isolates. SLS yielded a similar number of coelomycetes and hyphomycetes, although the hyphomycete assemblage was more expressive due to very frequent genera high abundance of occurrence (Figure 3.7 D). In all four types of foliar lesion were found exclusive genera, although this number was slightly higher in TDB lesions. These exclusive genera were mainly coelomycetes and were not directly related to the hyphomycete and coelomycete assemblages distribution previously reported. Thus, the exclusive genera in TDB and PP were mainly coelomycetes and included coelomycetes

genera 2, 3 and 4, *Stagonosporopsis*, *Pseudoconiothyrium*, *Parastagonospora*, *Bartalinia*, *Foliophoma* and *Phyllosticta*, while the exclusive genera in LLS and SLS included an equal number of coelomycete and hyphomycete genera, as well as other fungal taxa, such as *Graphiola* and *Teratosphaeriaceae* genus 1. Exclusive hyphomycete genera were found in all four types of foliar lesion and included hyphomycetes genera 2, 5 and 6, *Pithomyces*, *Monilia*, *Stachybotrys*. A total of eight genera were shared by all the foliar lesions types, including the very frequent genera *Alternaria*, *Cladosporium*, *Phoma* and other frequent and infrequent genera such as *Neosetophoma*, *Epicoccum*, *Penicillium*, *Stemphylium* and *Sordariomycetes* genus 1 (Figure 3.7 B).

Regarding TDB lesions it is likely that their occurrence is facilitated by abiotic factors that damage the tips of leaflets and segments, generating physical entries for the establishment of parasitic fungi. Coelomycetes seems to better colonize these lesions, where pycnidia development may be easier due to the existence of a previous damage tissue. This could also be associated with the fact that fungal richness per sample was higher in TDB lesions. In fact, the existence of a pre-damage tissue, either by abiotic or biotic (primary pathogens) factors, exposes plant tissue to easier colonization by secondary weakly parasitic microorganisms, which easily rupture and invade necrotic cells (Agrios, 2005).

Although four different foliar lesions types were defined as representatives of a set of morphological characters, differences between each foliar lesion can be observed and were evident. Considering that each foliar lesion analysed within each type represents a fungal community interacting with the diseased plant tissue, these differences were plotted and can be assessed in Figure 3.8. To plot these differences two components were considered, namely the diversity of the fungal community of each foliar lesion (expressed with their morphological and genetic indices of diversity and evenness) and the symptoms and signs observed as possible result of the growth of that fungal community (expressed through the size, the distribution, the presence of halo and the presence of fruiting bodies).

Component of symptoms and signs was more variable in TDB lesions and LLS, particularly in the presence or absence of halo and fruiting bodies, while component of fungal community was similarly variable in all foliar lesion types. Nevertheless, most of this variability lies within the indices of diversity between different foliar lesions within each type, since the evenness of the different fungal communities was shown to be very similar and high. Differences in the evenness values are more expressive in PP than the remaining foliar lesion types. In general, excepting certain cases, morphological and genetic indices of diversity showed a quite similar value in each foliar lesion, although genetic index of diversity tend to show higher values (Figure 3.8). This may indicate that if the isolates were identified to species level, a different pattern could be found for the composition of fungal communities.

How many isolates can be found in a single foliar lesion?

The fungal richness that can be found in a single foliar lesion was investigated using boxplots of the number of isolates against foliar lesion types and against the overall foliar lesions (Figure 3.9 A). The measure of central tendency used in the present analysis is the median and not the mean, since the first is less strongly affect by the extreme or discrepant values (outliers) on an asymmetric distribution (Zar, 2014). Discrepant values for the number of isolates were found in all foliar lesion types, but all of them were above the maximum value (Figure 3.9 A). These should be considered as extraordinary outliers, since they are so aberrant that lay at least 3 times the interquartile range from the box (Zar, 2014). TDB lesions boxplot showed a higher number of outliers than the other foliar lesion types (Figure 3.9 A).

The median number of isolates was quite similar in all foliar lesion types and in the overall sample (around 4 to 5 isolates). Nevertheless, evident differences in the dispersion of the number of isolates were found (Figure 3.9 A). In general, all foliar lesion types displayed a great variability in the number of isolates yielded. However, fungal richness in PP was more variable compared to the remaining foliar lesion types, while TDB samples were shown to be more homogeneous in their fungal richness (Figure 3.9 A, B).

None of the foliar lesion types data sets was symmetrical, however the skewness was different between TDB lesions and the remaining types. While TDB lesions presented a left-skewed data set, the remaining foliar lesion types and the overall sample presented a right-skewed data set, which means that 50% of the

samples yielded a relatively low number of isolates, and some of the remaining samples yielded much more than six isolates skewing the data to the right (Figure 3.9 A, B).

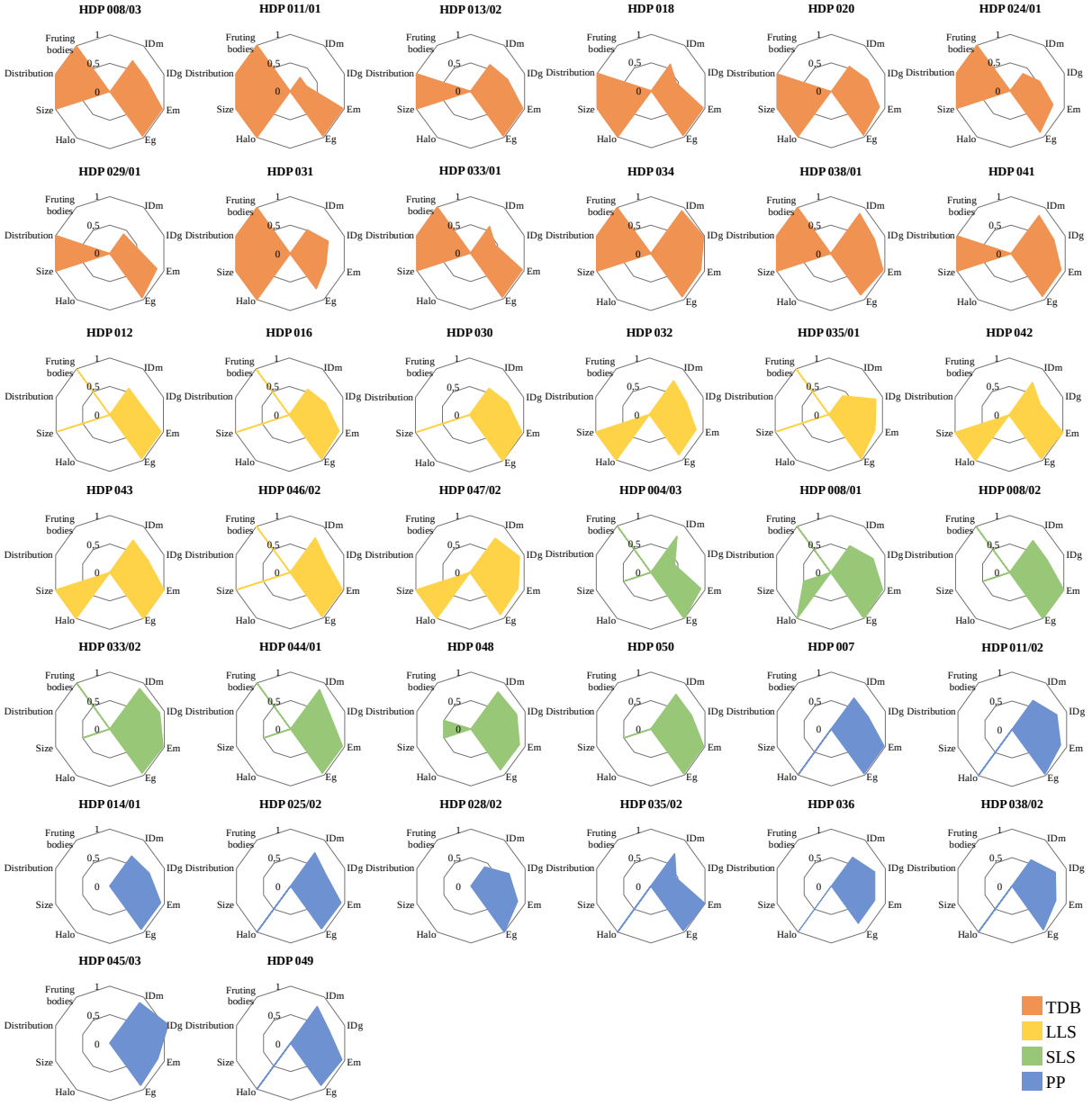

Figure 3.8 Graphical representation of the variation of each foliar lesion morphology and respective fungal community. Variation on foliar lesion morphology (left side of each graph) was assessed considering a three-values scale code (0, 0.5 and 1) for three different characteristics, namely the presence of fruiting bodies (no = 0, yes = 1), the distribution along the leaflet or segment (random = 0, specific region = 0.5, at the tip = 1) and the presence of halo (no = 0, yes = 1). Variation on the fungal community recorded in each foliar lesion (right side of each graph) was assessed through their morphological and genetic index of diversity and evenness, both computed using the Shannon index of diversity applied to the genera richness and its composition and MSP-PCR fingerprintings, respectively. Samples with a relative frequency of isolates < 1% (less than five isolates) were excluded. Colours are according to foliar lesion types. IDm = morphological index of diversity, IDg = genetic index of diversity, Em = morphological evenness, Eg = genetic evenness, TDB = tip die-back, LLS = large leaf spot, SLS = small leaf spot, PP = pinpoints and punctuations.

LLS, SLS and PP, as well as the overall sample, presented their median further from the middle of the boxplot, and an right tail much longer than the left tail, which also indicates that the distribution of the number of isolates for these foliar lesion types was skewed towards a higher number of isolates than the distribution

found for TDB (Figure 3.9 A), thus the number of isolates varies more widely in those types. The boxplot for TDB lesions is comparatively short, which suggests that the set of samples for this foliar lesion type yielded a relatively similar number of isolates, thus the distribution was less variable than for the remaining foliar lesion types (Figure 3.9 A, B). The left tail for TDB lesions was relatively longer, suggesting that the number of isolates for this foliar lesion type was more skewed towards a lower number of isolates, which results from the fact that most of the samples yielded around five to six isolates and all of the samples that yielded a number of isolates greater than six were outliers (Figure 3.9 A, B). Similarly, the boxplot for PP is comparatively long, which suggests that the number of isolates yielded from the set of samples for this foliar lesion type was highly variable, insomuch that only one sample was an outlier corresponding to the only sample that yielded more than 25 isolates (Figure 3.9 A, B).

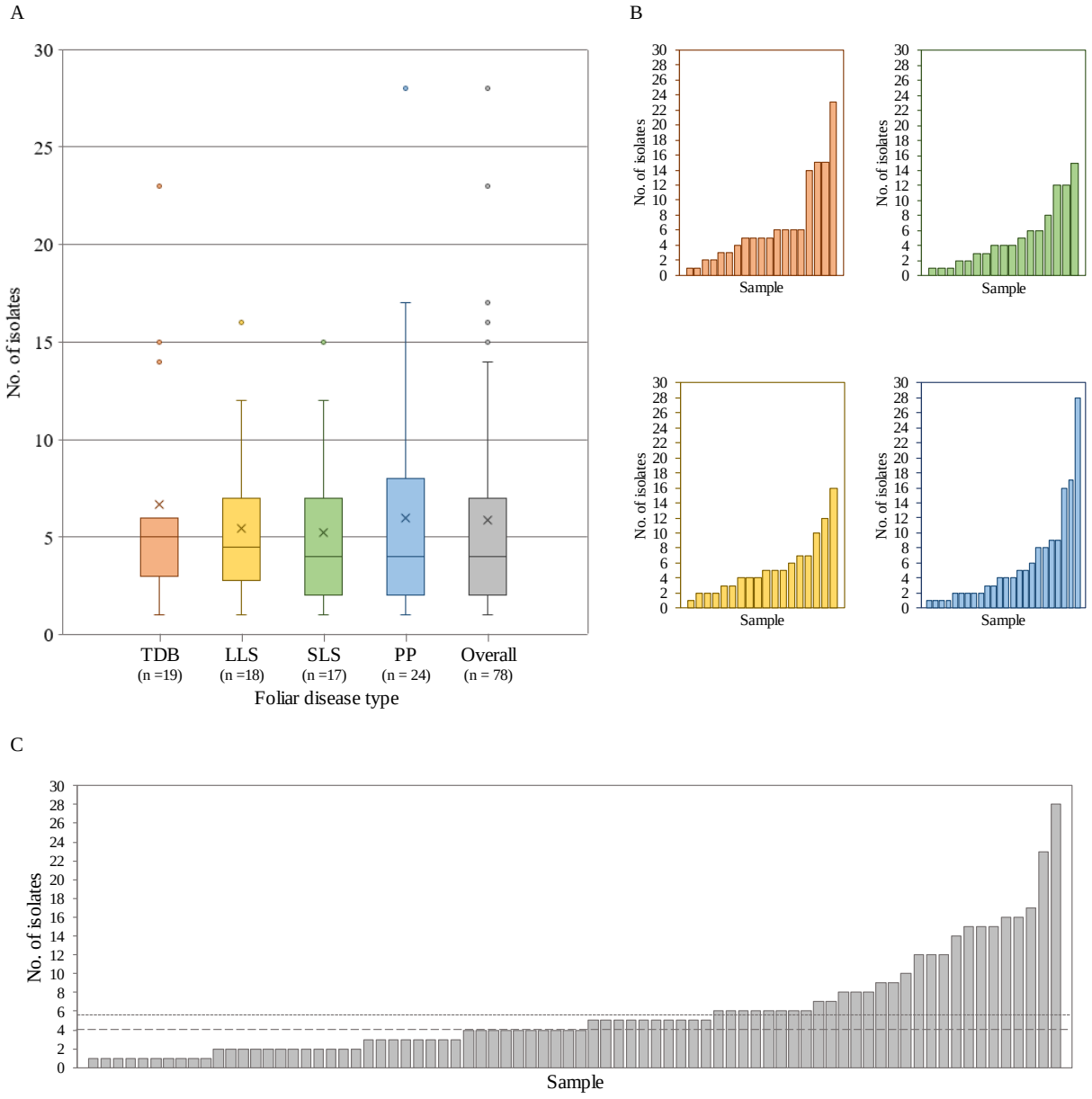

Figure 3.9 Distribution patterns of the fungal richness per sample according to foliar lesion type. **A**. Boxplots showing the distribution of the number of isolates per sample according to foliar lesion type. A boxplot for the overall set of samples was included. The lower and upper boundaries of each boxplot enclose 25–75% of the data. The line within the boxplots shows the median value, the bar lines above and below the boxplots indicate minimum and maximum values, × indicates the mean value and • indicates outliers. **B**. Number of isolates in each sample per foliar lesion type. Samples are ranked from the least to the highest fungal richness yielded. **C**. Number of isolates in each sample for the overall set of samples. Dashed line is relative to the median number of isolates. Dotted line is relative to the mean number of isolates. Colours are according to foliar lesion types. TDB = tip die-back, LLS = large leaf spot, SLS = small leaf spot, PP = pinpoints and punctuations.

Boxplot of overall sample was more similar to SLS boxplot than any other foliar lesion type boxplot, except for the maximum value, since some of the outliers in TDB lesions were not outliers when considering the distribution of the number of isolates for the overall sample. Nevertheless, no remarkable changes were observed in the boxplot for the overall sample compared with the distributions of each foliar lesion type (Figure 3.9 A). Considering the median and mean number of isolates (4 and 6, respectively) for the overall sample, the average number of isolates expected to be found in a single foliar lesion is here reported as five (Figure 3.9 A, C). However, the number of samples that yielded one, two, three, four, five and six isolates were approximately the same (Figure 3.9 C, Supplementary Table A.6).

Although the average number of isolates that is expected to be found in a foliar lesion of palms is five (Figure 3.9 A), different scenarios can be found when assessing the number of isolates recorded in a single foliar lesion. Further collections and isolation replicates would help to assess a more reliable value. Interestingly, the distribution pattern of the fungal richness per sample was remarkably similar in all four types of foliar lesions (Figure 3.9 A). This seem to indicate that the number of effectives that inhabit the foliar lesion is not entirely dependent on the composition of the fungal community and may be more related with the constrains mediated by the plant defences and the availability of nutrients.

Some samples presented discrepant values for the number of isolates, insomuch that a total of 15–28 isolates per sample was recorded. This was particularly relevant in samples from TDB and PP types.

In TDB lesions, the necrotic tissue was always largely spread through the leaves, and in some cases, they should probably be considered as dead material, which can explain the higher number of fungi that was reported from TDB lesions. The succession that phyllosphere fungal communities undergoes, particularly at phylloplane level, during the death of the leaves has already been showed (e.g. Stone, 1987; Osono, 2002; Voříšková and Baldrian, 2013). Frequently this succession encompasses an increase of fungal diversity, since after senescence, the phylloplane fungi readily gain access to the available nutrients in dead leaves biomass (Stone, 1987; Voříšková and Baldrian, 2013). Furthermore, after leaves senesce, certain taxa may change from endophytic to a saprotrophic life strategy, contributing for the increasing in fungal diversity (Osono, 2002; Koide *et al*., 2005; Promputtha *et al*., 2007; Voříšková and Baldrian, 2013). A similar scenario is expected to be occurring in highly necrotic LLS and SLS, where brittle tissue was found always to support an atypical high fungal richness per sample.

In PP, which were never found to present necrotic tissue, the high fungal richness detected in some samples may be due to the isolation of fungal communities from different foliar lesions, since in this case the concept of discrete units was difficult to apply. These foliar lesions were frequently minutely in size, dispersed along the leaves and aggregated in sets of several symptoms close together. Consequently, between the morphological types defined, PP was the most heterogenous, since it included several foliar lesions where the main morphological character was the presence of minute hardly defined spots. This heterogenicity was well expressed by its distribution patterns of the fungal richness per sample (Figure 3.9 A) and may have contributed to a high fungal richness in several samples, insomuch that the sample that showed the highest fungal richness (28 isolates) belongs to PP type.

Fungal genera "co-occurrence" and "phytopathogenic complex" concepts: a way of unveiling biotic relationships? Can they be applied? What are the limitations?

The fungal genera recorded from the fungal community associated with foliar lesions of palms presented substantial differences in their percentage abundance of occurrence. As mentioned previously, among the 57 genera recorded, three were regarded as very frequent (more than 10% abundance of occurrence), one was regarded as frequent (more than 5% to 10%), 15 were regarded as infrequent (more than 1% to 5%) and 38 were regarded as rare (less than 1%) (Table 3.2). Such percentage calculations and in turn converting them into different frequency groups may also help to verify whether a very frequent fungal genus also has a high frequency of co-occurrence with other fungal genera or not (Supplementary Table A.7).

Examination of 78 samples of foliar lesions of palms revealed that 10 samples showed any single fungal genus, 10 samples presented a single fungus inhabiting the foliar lesions examined and at least two fungal isolates were recorded and interpreted as fungal communities in all the other samples (Table 3.6,

Supplementary Table A.6), with most of them yielding around 2–4 fungal genera and the mean value for the number of genera per foliar lesion being around 4.

Considering that the fungal records were isolated from foliar lesions, one may ask if these fungal communities are phytopathogenic complexes. To evaluate this hypothesis, it would be necessary not only to explore the evolution of the leaf spotting fungal communities over time, but also to analyse the corresponding healthy tissue. In fact, to consider that the fungal communities associated with the foliar lesions of palms represent phytopathogenic complexes is taking the concept to far from what the present analysis allows.

Table 3.6 Distribution of fungal genera occurrences on foliar lesions of palms. Number of samples supporting n fungal genera records per sample.

Fungal genera records (n) per sample	Break-up of number of samples examined
n = 1	10
n = 2	18
n = 3	15
n = 4	14
n = 5	7
n = 6	5
n = 7	3
n = 8	5
n = 9	1

Most of the genera records found with a great representativity within the foliar lesions of palms, such as *Alternaria*, *Phoma* and *Cladosporium*, are commonly reported genera both as saprophytes and as leaf spotting fungi. Thus, to assume that these records are involved in a phytopathogenic complex, it would first be necessary to confirm their pathogenicity through pathogenicity tests. As pointed out by Broschat *et al*. (2014), this is a common issue for palms, especially ornamental palms, since there are several reports of potential pathogens isolated from diseased palm tissue, but no pathogenicity test have followed-up on healthy palms. For example, while *Cocoicola californica* and *Serenomyces* spp. are considered the primary pathogens of petiole or rachis blight, other fungal pathogens with an extensive host range beyond *Arecaceae* are often isolated from the diseased tissue, such as *Diplodia*, *Dothiorella*, *Fusicoccum*, *Macrophoma*, *Phoma* and *Phomopsis*, and regarded as potential pathogens, although their pathogenicity has not been tested (Broschat *et al*., 2014, 2015).

Potential plant pathogens and non-pathogens are a natural part of the palm environment at the phylloplane, so it is easy to isolate these fungi rather than the actual pathogen causing the observed symptoms. According to Agrios (2005), when plants are negatively affected by an environmental factor, such as low moisture, nutrient deficiency and air pollution, they are weakened and predisposed to infection by one or more weakly parasitic pathogens, what can be seen, for example, in the annual predisposition that plants show to *Alternaria* infections. Interestingly, this was one of the main genera found in the present study, which seems to corroborate the hypothesis that several fungal records are secondary minor pathogens and not the principal cause of the foliar lesions. In addition, it has been reported that chlorotic and necrotic tissue resulting from nutritional disorders of palms are often colonized by leaf spotting pathogens acting as saprobes or opportunistic pathogens. These fungi become established on the dead tissue, sporulate and the spores then spread to healthy palm leaf tissue (Broschat *et al*., 2014, 2015). Thus, the phytopathogenic complex concept has its limitations and should not be applied concerning the present analysis. In this sense, it seems more reasonable to evaluate fungal genera co-occurrences between different foliar lesions as a way of unveiling biotic relationships. The fungal co-occurrence concept was recently explored on palms in studies concerning *Nypa fruticans* at Brunei (Sarma and Hyde, 2018).

The percentage of abundance of occurrence and comparison of each genus occurring singly or in co-occurrence with other fungal genera is presented in Supplementary Table A.7. Of a total of 57 fungal genera occurring in this study only seven fungal genera occurred singly, namely *Colletotrichum* (in one

sample), *Neosetophoma* (in one sample), *Phoma* (in one sample), *Cladosporium* (in three samples), *Alternaria* (in one sample), *Didymocyrtis* (in one sample), *Teratosphaeriaceae* genus 1 (in one sample) and *Morinia* (in one sample). Of these seven fungal genera, only one fungal genus (*Teratosphaeriaceae* genus 1) never occurred with other fungal genera, although its percentage abundance of occurrence was too low (only one isolate was reported). The remaining fungal genera were mostly observed to have a co-occurrence with one or more than one fungal genus (Supplementary Table A.6). Thus 50 fungal genera had co-occurrence with other fungal genera in different combinations.

The most important examples of the co-occurrence shown by the samples in the present study were as follows (from the most to least abundant): (a) *Alternaria-Cladosporium-Phoma*; (b) *Alternaria--Cladosporium-Phoma-Epicoccum*; (c) *Alternaria-Cladosporium-Phoma-Epicoccum-Stemphylium*; (d) *Alternaria-Cladosporium-Stemphylium*; (e) *Alternaria-Cladosporium*; (f) *Cladosporium-Phoma*; (g) *Neosetophoma-Phoma*; (h) *Neosetophoma-Sclerostagonospora*.

Remarkable cases of fungal genera co-occurrence were positively related with fungal genera with high abundance of occurrence. *Alternaria* had co-occurrence with 45 of the 57 fungal genera recorded but on different samples and with different combinations, as outlined above, comprising from one fungal genus to a total of eight fungal genera co-occurring with *Alternaria* within a single foliar lesion. This pattern was also observed in other genera, such as *Cladosporium* which had co-occurrence with 39 fungal genera and *Phoma* which had co-occurrence with 34 fungal genera. This was followed by *Stemphylium* which had co-occurrence with 27 fungal genera and *Epicoccum* which had co-occurrence with 24 fungal genera. The remaining fungal genera showed co-occurrence with less than 25 but more than two fungal genera, excepting for the referred case of *Teratosphaeriaceae* genus 1 (Supplementary Table A.7). It is interesting to note that several infrequent and rare genera often co-occurred with several fungal genera, showing even a higher percentage abundance of co-occurrence than *Neosetophoma*, which was regarded as a frequent genus. *Neosetophoma* had co-occurrence with 18 fungal genera, a smaller number comparing with infrequent genera such as *Stemphylium*, *Sordariomycetes* genus 1 and *Epicoccum*. In addition, the presence of *Neosetophoma* was often followed by the absence of *Cladosporium*, although this was one of the genera with higher percentage of abundance of occurrence, frequency of occurrence and frequency of co-occurrence (Supplementary Table A.7).

Interactions between organisms and environmental effects on co-existence within biological communities are often explored in ecology through co-occurrence patterns (Williams *et al*., 2014). These co-occurrence patterns can reflect the ecological processes that drives the coexistence and diversity maintenance within biological communities (HilleRisLambers *et al*., 2012).

In the present study, *Alternaria* (21% of all records), *Cladosporium* (12%) and *Phoma* (10%) which were very frequently recorded genera, occurred both with other fungi and singly. However, their occurrence singly was minimal when compared to their frequency of co-occurrence. This may indicate a mutualistic association. Previous studies have shown that under controlled condition, dominant fungi, especially primary decayers, influence other fungi growing together with them. For example, species richness and composition of fungal communities were shown to be affected by *Fomitopsis pinicola* in *Picea abies* logs recovered from an old-growth mountain spruce forest in the Bohemian Forest, Czech Republic (Pouska *et al*., 2013). A similar pattern was recorded on *Avicennia officinalis* colonized by the dominant fungus *Lignincola laevis* in a south-western mangrove of India (Maria & Sridhar, 2017). More recently, a similar pattern was reported for *Linocarpon bipolaris* and *L. appendiculatum* colonizing *Nypa fruticans* in Tutong River, Brunei (Sarma and Hyde, 2018). Thus, it can be hypothesized that isolates of *Alternaria*, *Cladosporium* and *Phoma* were not only the dominant colonizers of foliar lesions of palms, but also accommodative for other fungi with which they live in a mutualistic association. The present analysis does not allow to predict if the isolates of these genera are dependent on other fungi, as a fungal community, to colonize the foliar lesions, i.e., to infect and damage the cells of the leaves. Only laboratory experiments will unveil if these isolates are phytopathogenic or need others to create suitable conditions for their subsequent colonization. Furthermore, these laboratory experiments may unveil other biotic relationships, such as competition and antagonism, which may exclude other fungi from colonization. For example, previous studies by Fryar *et al*. (2001, 2005)

revealed *in situ* and *in vitro* patterns of competitive hierarchy between fungal species living on submerged wood at Hong Kong and Brunei.

Only 7 fungal genera were found to occur singly. Nevertheless, their percentage abundance of occurrence singly was far too low to attribute an antagonistic potential in preventing other fungi from colonization. In addition, some of these genera were regarded as very frequent or frequent. Again, to predict these biotic relationships, it must be proven through *in vitro* experiments whether these particular isolates have some antagonistic potential. Sarma and Hyde (2018) found a similar pattern of single occurrences on a survey from *Nypa fruticans* at Brunei, and no prediction in terms of antagonism relationship was undertaken due to low fungal percentage of occurrence. However, other studies, such as those of Sarma and Raghukumar (2013) in manglicolous fungi from Goa, had reported antagonistic lifestyle based on a high percentage of singly occurrences. Although in the present study, it cannot be assumed that the singly occurrences were due to an antagonistic lifestyle, an interesting pattern was observed between the occurrences of genera *Cladosporium* and *Neosetophoma*. These two genera rarely occurred together, even though they were regarded as very frequent and frequent, respectively. It is likely that one of these genera (or both) presents antagonistic potential and *in vitro* experiments should be undertaken to evaluate it.

As referred, almost all the genera that occur singly and that were regarded as very frequent or frequent, presented a much higher percentage frequency of co-occurrence. Thus, none of these genera can be considered commensals, i.e., fungal genera which their association with other genera is not necessarily dependent on others. Hyde and Sarma (2018) pointed out that three species, *Oxydothis nypae*, *Astrosphaeriella striatispora* and *Linocarpon nypae*, could be considered commensals in the fungal communities recorded from *Nypa fruticans* due to the equal proportion observed in their occurrences singly and with other fungi. A similar pattern was also observed in those referred studies by Sarma and Raghukumar (2013).

3.5. Overall genetic diversity: what degree of fungal genomic diversity is there?

An analysis based on csM13 and $(GTG)_5$ MSP-PCR genomic fingerprintings profiles was done to assess genetic diversity within the collection of isolates established from diseased palm leaves. This approach yielded highly reproducible and complex genomic fingerprints, with several bands ranging from 200 to 3500 bp. Based on these genomic fingerprints, all 457 isolates were clustered in a consensus dendrogram (data not shown). A review of the available literature suggests that genomic DNA fingerprints have never been applied to evaluate genetic diversity within fungal communities from palms.

The reproducibility system: towards a concept of isolation redundancy?

A 95% conservative cut-off level was established, above which isolates cannot be discriminated using this technique. This was based on the reproducibility level calculated according to the percentage similarity between the duplicates (Supplementary Figure A.6). The fingerprints obtained with csM13 were in general more complex and discriminatory than those obtained with the $(GTG)_5$ primer. Nevertheless, the combined analysis of both profiles presented a higher discriminatory power and allowed to resolve some clusters that were not discriminated at genus level when the primers were used individually. This was expected, since the discriminatory power of each primer is dependent on the taxonomic group that is being analysed and polyphasic approaches usually present a greater capacity for differentiation among microbial isolates.

The overall clustering pattern allowed to determine an isolation redundancy rate around 6%. This was calculated considering the isolates of the same genus and from the same foliar lesion that paired above the 95% similarity cut-off level. These isolations were considered redundant, since they probably corresponded to independent isolations of the same organisms inhabiting a certain foliar lesion. The value found for the isolation redundancy rate was considerably low, accounting only 27 isolates. Furthermore, most of these isolates were from a single genus, *Alternaria*. Thus, it is possible to assume that the previous ecological observations pointed out were effectively based on different isolates and that the associated error was negligible. Even considering that some of the isolates of *Alternaria* came from redundant isolations, the number of different isolates of *Alternaria* recorded continue to demonstrate that this genus was the most

frequently and abundantly found in the fungal communities surveyed. Thus, isolation redundancy rate calculation based on genomic fingerprints seems to be an interesting tool to apply in ecological studies. With the appropriate molecular markers, isolation redundancy rate can function as a fine-tuning tool while analysing the structure of fungal communities.

Genetic diversity patterns: are genomic types different between host species and parishes?

To further analyse the genetic diversity patterns, new dendrograms were constructed for subsets of isolates grouped according to a characteristic of interest, such as host species or parishes where they were isolated from, and fungal genera to which they belong (data not shown). Only fungal genera with a percentage abundance of occurrence of at least 1.5% were considered in the present analysis to avoid any biased result due to lack of information. It was possible to verify that genomic types (clusters) for each genus encompass a wide distribution of different host species and parishes. Thus, as a preliminary result, apparently there was no association between genomic types per genera and their distribution among host species or parishes. Nevertheless, further sampling may be able to unveil a different pattern, especially for genera regarded as rare, since these were excluded from the present analysis.

The results obtained through these subsets allowed to compare the genetic biodiversity patterns of fungal communities with those obtained for the morphological analysis. The genetic diversity was calculated using the Shannon index of diversity, considering a cut-off level of 70% similarity to generate clusters. Results obtained were then compared with the same calculations made for the morphological analysis (Figure 3.10). This was already presented and discussed in the previous subsections (3.1, 3.2, 3.3 and 3.4) and the calculations were made considering fungal richness and their composition in terms of number of isolates.

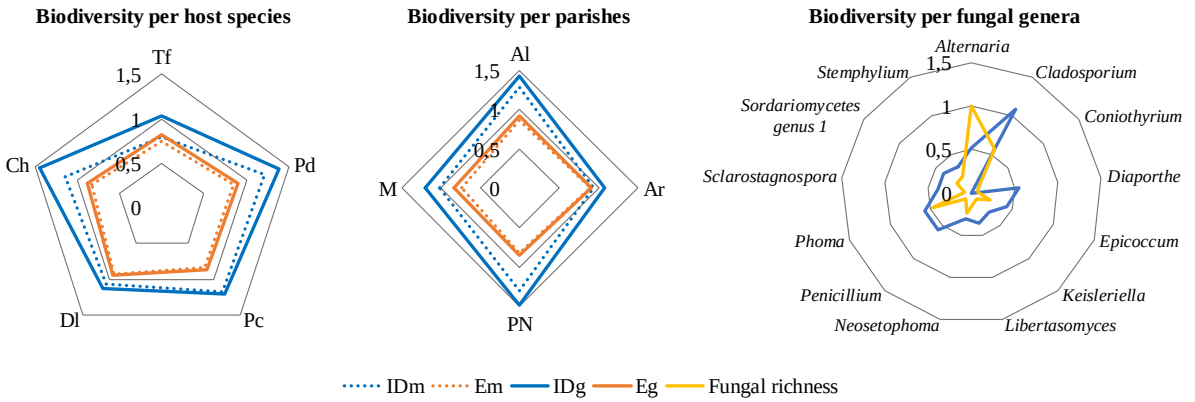

Figure 3.10 Graphical representation of morphological and/or genetic biodiversity patterns per host species, parish and fungal genera. Biodiversity patterns of the fungal community surveyed from each host species, parish and fungal genus was assessed through their morphological and/or genetic index of diversity and evenness, both computed using the Shannon index of diversity applied to the genera richness and its composition and MSP-PCR fingerprintings, respectively. IDm = morphological index of diversity, IDg = genetic index of diversity, Em = morphological evenness, Eg = genetic evenness, Dl = Dypsis lutescens, Tf = Trachycarpus fortunei, Pd = Phoenix dactylifera, Pc = Phoenix canariensis, Ch = Chamaerops humilis, Al = Alvalade, Ar = Areeiro, PN = Parque das Nações, M = Marvila.

It was verified that in all host species, as well as in all parishes, genetic diversity was as high or higher compared to the morphological diversity (Figure 3.10). This was particularly relevant in *Chamaerops humilis* and *Trachycarpus fortunei*, which is coherent with the previous analysis concerning the morphological diversity. In fact, these two hosts species are typical temperate climate palms and their climatic constrains and requirements seem to be playing a role in the genetic diversity of fungal communities. The relative higher genetic diversity found on *C. humilis* and *T. fortunei* may be associated with the fact that these palms are adapted to temperate climate and, subsequently, to its typical mycobiota. In addition, their ability to share fungal isolates with other typical temperate hosts may increase the potential genetic diversity among the fungal communities. Contrastingly, *Phoenix canariensis* and *Dypsis lutescens* presented a genetic diversity almost equal to the morphological diversity previously assessed. This suggests that genera within their fungal communities were quite homogeneous concerning their genomic fingerprints. Considering that these two

host species are typical tropical and subtropical palms, their introduction into temperate climates may lead to a decrease on the diversity of their fungal communities.

In all parishes, the genetic diversity was also higher than the morphological diversity. In Parque das Nações and Alvalade, the genetic diversity was found to be particularly high, what is according to the previous observations regarding the morphological analysis. Thus, the greater availability of moisture in the sampling sites within these two parishes seems to also influence the genetic structure of the fungal communities. Since moisture positively influences fungal proliferation and sporulation, it is not surprising that the genetic structure within these two parishes were found to be much more variable and complex. Although differences between genetic and morphological biodiversity patterns were found, genetic and morphological eveness were quite similar. This suggests that genomic types within fungal communities are similarly well distributed between the different genera (Figure 3.10). Moreover, biodiversity differences within the fungal communities seems to rely more on the genomic types present (which are directly related to the genetic diversity) than on their relative distribution.

Morphological traits and cultural characteristics have been often difficult characters to use in the differentiation of strains of filamentous fungi (Meyer *et al*., 1993a). In the present analysis the morphological characters have been used to identify the isolates to genera, but within the same genus, excluding particular cases, it was not possible to reach further differentiation. In this sense, genomic fingerprints are an exceptional tool for microbial genotypic characterization and, subsequent, differentiation (Jeffreys *et al*., 1985; Rademaker and de Bruijn, 1997). The discriminatory power, as well as high reproducibility, of MSP-PCR (Olive and Bean, 1999) was well evident in the present analysis. In fact, the fungal communities of diseased palm leaves revealed a remarkable high level of genetic diversity, which allowed to differentiate isolates with very similar micromorphology. However, the contribution of different genera for the genetic diversity of fungal communities was distinct (Figure 3.10, Figure 3.11). For example, while *Alternaria* was found to be highly homogeneous concerning its genomic profiles, *Cladosporium* was found to be highly diverse. This is particularly substantial considering that the fungal richness for *Alternaria* was much higher than the fungal richness for *Cladosporium*. One would expect that the analysis of a larger number of isolates would support greater genetic diversity, nevertheless the present analysis suggests that this may not be true. These patterns and small nuances show how complex ecological analyses can be when several approaches are taken into consideration.

Only an integrated approach with both morphological and molecular data may reveal in full how microbial communities are shaped. Considering the three most abundant genera here recorded, i.e., *Alternaria*, *Cladosporium* and *Phoma*, it is clear that biodiversity within fungal communities relies on much more factors than only the richness of genera and fungal isolates. Most isolates of *Alternaria* recorded were found to cluster within the same genomic type (cluster I, Figure 3.11 A), which probably represents different strains of the same species. A similar pattern was found in *Phoma* isolates, since most of them also fall within the same genomic type (cluster V, Figure 3.11 B). However, a very distinct pattern was found in *Cladosporium* isolates, where a greater number of genomic types were found (clusters I–XI, Figure 3.11 C) harbouring, in general, a more or less similar number of isolates. Thus, considering the distribution of the number of isolates per cluster, the genetic structure of *Cladosporium* assemblage seems to be much more even than what was recorded for *Alternaria* and *Phoma*. These last two genera were found to be much more homogeneous regarding their genetic diversity. If these differences are related with the presence of several species or only with the existence of a wide range of genomic variation among *Cladosporium* isolates cannot be predicted with the present study. Similarly, it cannot be assumed that *Alternaria* and *Phoma* assemblages are composed by mainly one species, but mainly a certain genomic type. Further studies including genomic profiles of reference strains or through sequencing of appropriate DNA barcodes for representative isolates may clarify in deep the ecological and biodiversity patterns here observed.

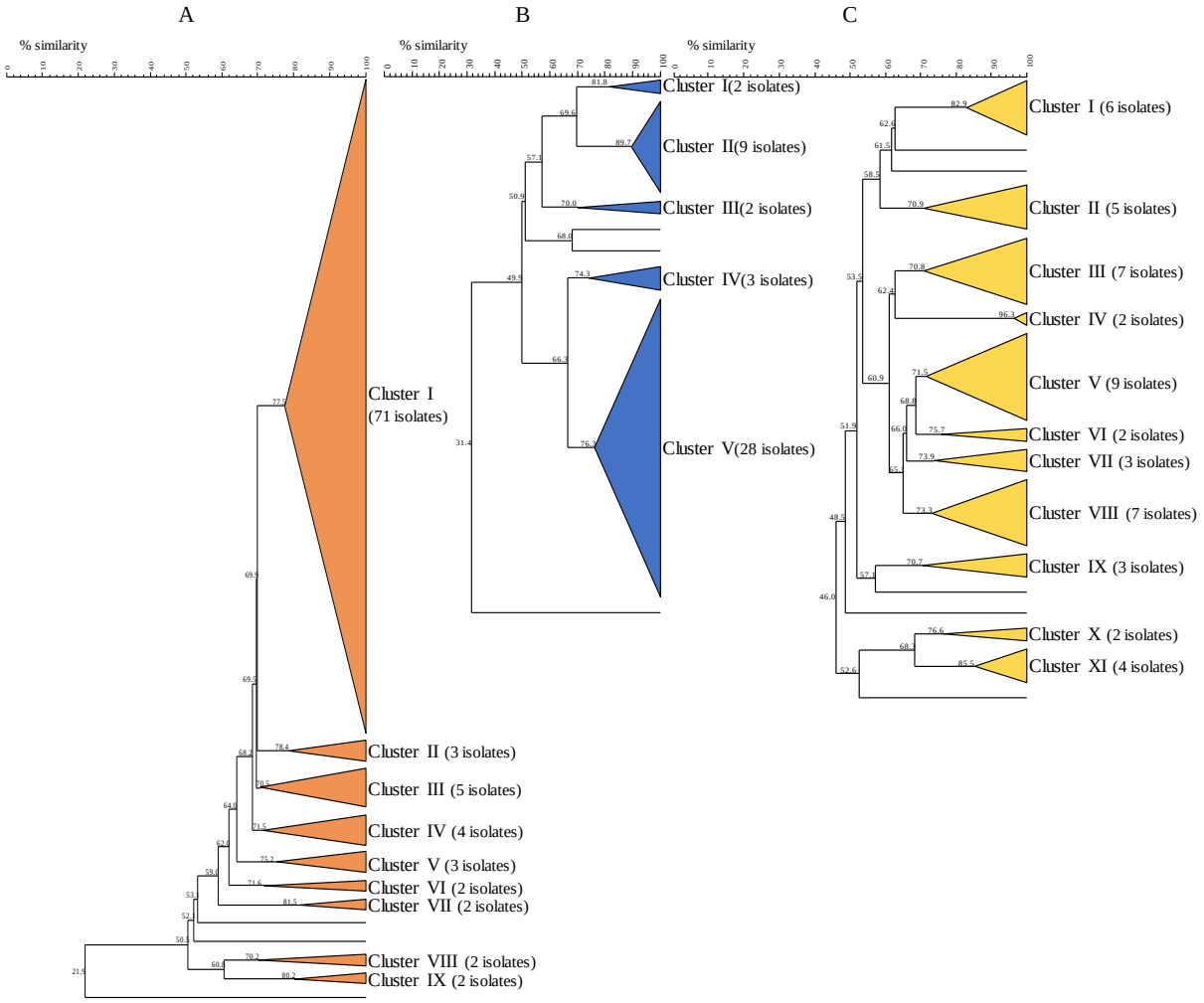

Figure 3.11 Genomic diversity among the most abundant fungal genera recorded. Simplified composite dendrograms at a cut-off level of 70% to generate clusters (coloured triangles). Dendrograms were based on csM13 and $(GTG)_5$ MSP-PCR profiles performed in BioNumerics using Pearson's correlation coefficient and UPGMA. **A**. Simplified dendrogram for *Alternaria* isolates. **B**. Simplified dendrogram for *Phoma* isolates. **C**. Simplified dendrogram for *Cladosporium* isolates.

Besides the differences regarding fungal genera and fungal communities surveyed from different palm species and parishes, differences for the genetic diversity within hyphomycetes and coelomycetes were also observed (data not shown). The genetic diversity within coelomycetes assemblage was much higher than that found within hyphomycetes assemblage, comprising 28 clusters highly coherent with the morphological analysis and highly even (from 2 to 11 isolates per cluster, except for a cluster of *Phoma* that comprised 28 isolates). Hyphomycetes assemblage comprised 26 clusters, but the isolates distribution between clusters were highly uneven, since 95 out of 243 isolates fell within the same cluster, where almost all *Alternaria* isolates were found. In addition, several of these clusters were not coherent with the morphological analysis used to identify the isolates at genus level. This may suggest that the molecular markers used are more appropriate to discriminate coelomycete than hyphomycete genera, since the congruence between fingerprinting profiles and taxonomic positioning was remarkable among coelomycete genera. It is to be noted that coelomycete assemblage presented a much broader taxonomic range within fungal families than hyphomycetes and this may also justify the congruence found in fingerprints. A broader taxonomic range certainly implies phylogenetic differences that may be also expressed in the target repeated sequences used to assess the genomic profiles.

A subset of the overall coelomycetes dendrogram can be seen in Figure 3.12. This subset shows clearly the already mentioned congruence between fingerprinting profiles and taxonomic positioning. The extent of this congruence was observed even within the same genus. For example, two clusters were observed for

Neosetophoma isolates (*Neosetophoma* I and *Neosetophoma* II, Figure 3.12), which were coherent with morphological differences observed in spores septation and colour.

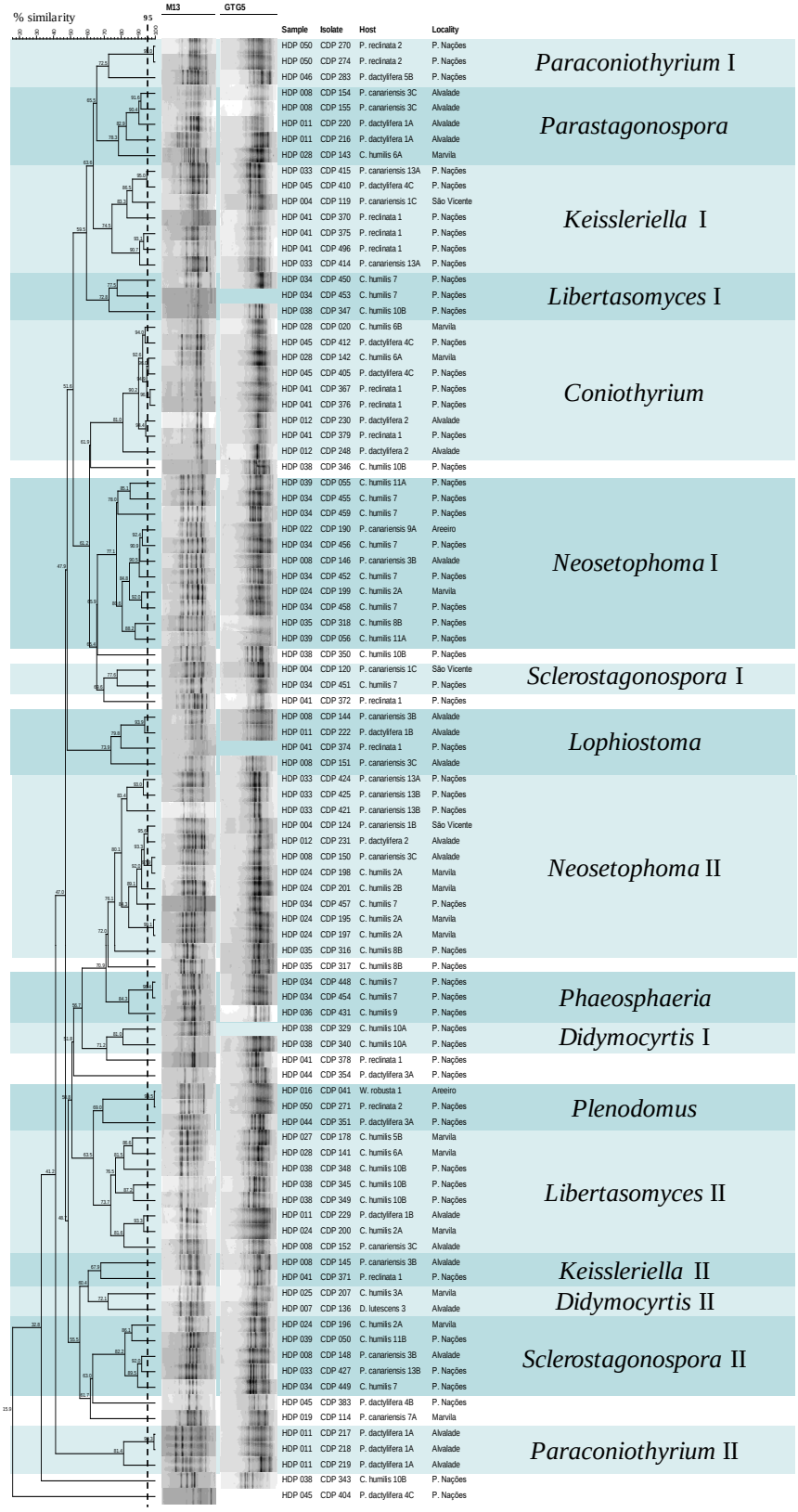

Figure 3.12 Genomic diversity among coelomycetes assemblage. Composite dendrogram based on csM13 and $(GTG)_5$ profiles performed in BioNumerics using Pearson's correlation coefficient and UPGMA. The vertical dashed line corresponds to the reproducibility level (95%). Clusters coherent with the morphological analysis are coloured and named with the corresponding genus, which was assessed through sequencing of ITS region and subsequent BLAST against GenBank database.

Genomic fingerprinting was the technique used in the present study to identify highly morphological similar coelomycetes to genus level. For this purpose, representative isolates from each cluster formed around 60% to 70% similarity and, when possible, isolates from different parishes and palm species within the same cluster were selected for sequencing of ITS region. Figure 3.12 presents the result obtained from this preliminary analysis, and the closest-hit genus and species for each isolate sequenced can be accessed in Supplementary Table A.13.

Some of the isolates here sequenced as a preliminary approach were selected for in-depth analyses to determine if they account for some of the undescribed global mycota (see Part II).

4. Global Discussion and Final Remarks

Biodiversity measures are highly dependent on the sampling effort, as well as on all subsequent analyses and methods used to study the fungal assemblages. Therefore, the present approach should be considered as a snapshot assessment of the biodiversity of palm leaf spotting fungal communities, and not as a statistically validated model; nonetheless, in this work, the foliar lesions have been studied as fungal communities. In this sense, several biodiversity and ecological tendencies have been pointed out, although most of them have not been subjected to statistical.

Several questions arose as the main objectives of this approach, and the obtained answers are here presented and summarized, along with other considerations.

The diversity trends

No typical palm fungi genera (i.e., *Oxydothis*, *Anthostomella*, *Astrocystis*, *Astrosphaeriella*, *Linocarpon*, *Neolinocarpon*, *Fasciatispora*, and *Capsulospora*) were recorded in the present study. Instead, ubiquitous and common temperate genera were found. These included *Alternaria*, *Phoma* and *Cladosporium* as the main assemblages, followed by a plethora of less representative genera. The fungal community pattern was well-evidenced by the genera abundance distribution plot, which can be well-fitted by a log-series model. For this, coelomycete genera presented a greater contribution, with a higher number of infrequent and rare genera. Taxonomically, the fungal community had a broad distribution, with the most represented order being *Pleosporales* and *Capnodiales*. Except for *Graphiola*, a basidiomycete exclusively found on palm hosts (Piepenbring *et al*., 2012), all isolates belong to phylum *Ascomycota*. This result is in accordance to previous works on palm fungi (Fröhlich and Hyde, 2000; Hyde *et al*., 2000; Taylor and Hyde, 2003). This result was in line with the studies on temperate palms by Taylor *et al*. (2000) concerning the relative proportion of hyphomycetes and coelomycetes inhabiting palm tissues. This was particularly interesting, since these results were found in different contexts. Nevertheless, this analysis was based on a relatively small number of samples, and the conclusions obtained may be biased due to sampling effort. Future studies should aim further sampling, to establish more supported results about the fungal community patterns that inhabits palm foliar lesions in Portugal.

A matter of host and parish

No specific and recurrent pattern between fungal genera and different palm hosts or parishes was observed. Nevertheless, several ecological and biodiversity trends were detected. Relative humidity played an important role on fungal diversity. This was particularly evident in Parque das Nações and Alvalade. In addition, climate requirements were found to be important in shaping fungal richness in different host species. Tropical palms, namely *Dypsis lutescens* and *Phoenix canariensis*, presented a depauperate mycobiota when compared to the temperate palms *Chamaerops humilis* and *Trachycarpus fortunei*. No wild representatives of palms were studied, and the fungal assemblages recorded may reflect this. Although climate requirement is an influential factor, a survey of the fungal assemblage of wild representatives of warm temperate palms in Portugal, would be essential to establish a well-supported data pattern. Future studies should aim a systematic survey of the mycota from wild stands of the temperate palm *C. humilis*.

Co-occurrence in foliar lesions

In this study, a mean of 5 isolates per palm foliar lesion was found. This number seems to be independent of the lesion type. The found isolates were evaluated through their patterns of co-occurrence. These patterns can be used as a tool to unveil certain biotic relationships, irrespective of the fact that there are many factors that may influence these co-occurrences, and that most of them were not considered in this approach. In fact, as previously discussed, the host species as an environment, and the moisture availability, highly affect the composition and diversity of fungal communities. Thus, it would be noteworthy to verify if these patterns are different when analysing fungal communities from different host species, and from different parishes. The present study provides preliminary data on the co-occurrence patterns on foliar lesions of palms in Portugal. Future *in vitro* studies, i.e., culture dependent experiments, may unfold more specific fungal interactions, such as mutualism, commensalism, and antagonism. Moreover, the patterns of co-occurrence in the samples examined may be further clarified if the isolates are identified to species level.

An (in-)adequate sampling size

This study revealed a high fungal diversity inhabiting foliar lesions in palms. Nevertheless, biodiversity analyses through the combined application of genera-accumulation curves, and the Chao1 species richness estimator, predicted that just over 50% of the potential leaf spotting fungal mycobiota was found. Although the accessed curves did not level-off, the number of samples was large enough to obtain representative results that expressed certain ecological and biodiversity trends. The remaining genera to be recorded are expected to be infrequent and rare genera, since it was clear that the communities followed a log-series model, with a long tail of infrequently recorded genera. Possibly, a lot of these genera will be coelomycetous fungi, given that the assemblage of this fungal type comprised most of the infrequent and rare genera, and are the main assemblage that contributes for the high fungal diversity reported.

A genetic diversity through genomic traits

Differences in the morphological and genetic diversity of the fungal communities of each foliar lesion were evident. In general, as expected, genetic diversity was found to be higher than morphological diversity, since genomic analyses allow to discriminate isolates in the same genus (Meyer *et al*., 1993a). Genomic fingerprinting profiles highlighted the future need to identify the isolates at species level. In fact, genomic types revealed that the biodiversity patterns for some genera are highly complex. Consequently, reducing these genera to a single 'morphogroup' may lead to misinterpretations on the biodiversity structure that lies on the foliar lesions of palms.

The reliability of diversity indices

Several biodiversity patterns were studied and evaluated taking into consideration diversity indices, especially Simpson and Shannon indices of diversity. These indices were more efficient in revealing differences when applied to the coelomycete and hyphomycete assemblages, than when applied to different host species and to different parishes. This result was expected, as it relies on the sample size that was used in each analysis. Gimaret-Carpentier *et al*. (1998) showed that Simpson index of diversity reaches a stable value at low sample sizes, while Shannon index of diversity is more affected by the addition of rare species with increasing sample size. Both aspects can be seen in the present study. Simpson index of diversity rarely revealed differences between the fungal assemblages of different hosts or parishes but was reasonably efficient in revealing diversity differences between the coelomycete and hyphomycete assemblages. This occurred because the dominance of the very frequent and frequent genera rapidly stabilized the value of the diversity index in small-sized samples. In this case, the Simpson index of diversity becomes blind to the addition of rare genera and assumes a value that is close to the relative abundance of the dominant ones. A similar effect is observed in the Shannon index of diversity, although it usually performed better in revealing differences. This occurs because the main differences in the fungal assemblages recorded rely in the infrequent and rare genera, especially those of coelomycetes. Both diversity indices are sensitive to genera

abundance distribution but give different weigh to rare genera. Thus, Shannon index of diversity seems to be more adequate to evaluate the diversity of the leaf spotting fungal community, since it follows a log-series distribution model, becoming rich due to the high profusion of infrequent and rare genera. The integrated analysis of the diversity indices also highlights the fact that coelomycete assemblage may be the main palmicolous fungal community in temperate regions, since the hyphomycete assemblage was composed mainly by highly abundant and plurivorous genera.

The results of this study are preliminary observations of the fungal communities on foliar lesion from different palm species and parishes in Lisbon, Portugal. Only the palms at some parishes were examined. If palms from other sites were included, the results might be more conclusive. These preliminary results, do however, show that fungal communities on foliar lesions of palms are diverse and dynamic ecological units with several biodiversity patterns. This is consistent with the large number of fungi known to occur on palms and may account for some of the missing fungal diversity, which is the scope of Part II of this work.

PART II

Case studies on microfungi from palm foliar lesions: sizing up potentially new taxa

Case study I. *Diaporthe chamaeropsicola* sp. nov., a new *Diaporthe* species from palms in Portugal

Case study II. *Morinia trachycarpae* sp. nov. and *Morinia phoenicicola* sp. nov., two new *Sporocadaceae* species from palms in Portugal

Case study III. *Arecamyces humilianae* gen. *et* sp. nov., a new *Teratosphaeriaceae* genus and species from palms in Portugal

Case study I. *Diaporthe chamaeropsicola* sp. nov., a new *Diaporthe* species from palms in Portugal

1. Introduction

Diaporthe Nitschke, syn. *Phomopsis* (Sacc.) Sacc. (Saccardo, 1905), species are known as important plant pathogens, endophytes and saprobes on a variety of economically important crops, ornamentals and trees (Santos and Phillips, 2009; Santos *et al*., 2011, 2017; Udayanga *et al*., 2011, 2015; Huang *et al*., 2013; Hyde *et al*., 2014; Dissanayake *et al*., 2015, 2017a,c; Guarnaccia *et al*., 2018). Along with its diverse host ranges and worldwide distribution, the interest in this genus has grown over the years due to its recurrent association with plant diseases. Common diseases caused by *Diaporthe* species include leaf spots, blights, seed decay, cankers, dieback and wilt (Mostert *et al*., 2001a,b; van Rensburg *et al*., 2006; Diogo *et al*., 2010; Santos *et al*., 2011; Thompson *et al*., 2011, 2015; Diaz *et al*., 2017; Manawasinghe *et al*., 2019). Since the implementation of international phytosanitary measures relies on the correct identification of the phytopathogenic fungi (Santos and Phillips, 2009; Udayanga *et al*., 2011; Wingfield *et al*., 2012), the taxonomy of *Diaporthe* has often been re-evaluated to construct a natural framework. In the last decade, four major taxonomic revisions of the taxonomy of genus have been published (see Gomes *et al*., 2013; Gao *et al*., 2017; Dissanayake *et al*., 2017b; Marin-Felix *et al*., 2019b). However, each study has used different combinations of gene loci to resolve species boundaries.

The genus *Diaporthe*, based on *Diaporthe eres* Nitschke collected in Germany from *Ulmus* sp., was introduced by Nitschke (1870) and resides in the family *Diaporthaceae*, order *Diaporthales*, in class *Sordariomycetes* (Maharachchikumbura *et al*., 2015, 2016). For many years taxonomy of *Diaporthe* species was based on host association (Uecker, 1988; Udayanga *et al*., 2011), which led to a proliferation of species names. Although several species are known to be host-specific, most can be found on more than one host (Mostert *et al*., 2001a; Santos and Phillips, 2009; Diogo *et al*., 2010; Udayanga *et al*., 2014a,b; Guarnaccia *et al*., 2016). The genus *Diaporthe* is highly complex, comprising several cryptic species, many of which are well-described, and their phylogeny well-resolved (e.g. Gomes *et al*., 2013; Udayanga *et al*., 2014a,b, 2015; Guarnaccia and Crous, 2017). Since morphology is of limited value in defining species (Hyde *et al*., 2011), currently the circumscription of *Diaporthe* species relies on molecular phylogenies based on different loci. The most used loci for *Diaporthe* species include the rRNA region (ITS) and partial sequences of the translation elongation factor 1-alpha (TEF1), the β-tubulin (TUB2), the calmodulin (CAL) and the histone H3 (HIS3) genes (Udayanga *et al*. 2012a,b, Gomes *et al*. 2013; Hang *et al*., 2013; Tan *et al*., 2013; Hyde *et al*., 2014; Gao *et al*., 2017; Yang *et al*., 2018; Hyde *et al*., 2019a). All the older species names in *Diaporthe* or *Phomopsis* for which cultures and DNA sequence data are not available, cannot be considered to be reliable, and should be disregarded until they are re-collected and epityfied. The currently accepted *Diaporthe* species and their respective DNA barcodes can be assessed on Marin-Felix *et al*. (2019b).

A survey of the literature suggests that no intensive study with proper molecular data has been carried out to resolve the complex of *Diaporthe* species occurring on palms. Although several *Diaporthe* species have been described from palms, most of them were based mainly on account of their unique palm hosts but without molecular data to confirm their phylogenetic position. Subsequently, most of these species have not been transferred to *Diaporthe* and remain in *Phomopsis* (Fröhlich *et al*., 1997; Taylor and Hyde, 2003). Nevertheless, this genus name is no longer used since dual nomenclature for pleomorphic fungi was abolished (Rossman and Samuels, 2005; Santos and Phillips, 2009; Rossman *et al*., 2015). Fröhlich *et al*. (1997) provided a synopsis of *Diaporthe* (as *Phomopsis*) species known from palms and several other species have been reported by Taylor and Hyde (2003). Herein, a new synopsis of *Diaporthe* species from palms is presented considering the currently accepted and phylogenetically validated *Diaporthe* names.

In this study, one new species of *Diaporthe* associated with foliar lesions of palms in Lisbon, Portugal, *Diaporthe chamaeropsicola* DRS Pereira & AJL Phillips, is described based on morphological characters and phylogenetic data derived from sequences of ITS, TEF1, TUB2 and CAL. Furthermore, four new records of *Diaporthe* species from palms are presented. These include two new records from *Arecaceae* for the first time with the isolation of *D. foeniculina* (Sacc.) Udayanga & Castl. and *D. pyracanthae* L Santos & A Alves

from *Chamaerops humilis* and *D. foeniculina* from *Trachycarpus fortunei*, along with the isolation of *D. pseudophoenicicola* RR Gomes, C Glienke & Crous from *C. humilis*.

2. Materials and Methods

A schematic overview of the workflow used in the present work is presented in Supplementary Figure A.1.

2.1. Specimen collection and examination

Diseased palm leaflets and leaf segments were collected from Oeiras and Lisbon during September and October 2018. Specimens were transported to the laboratory, examined with a Leica MZ9.5 stereo microscope for observations on lesion morphology and for the presence of fungi. Morphological details of lesions were observed on both adaxial and abaxial surfaces.

2.2. Culture media and growth conditions

Cultures were grown on 1/2 PDA and, unless stated otherwise, incubated in ambient light at room temperature (18–20 ℃). To stimulate sporulation, isolates were cultured on 2% WA with healthy doubled autoclaved *Populus* sp. twigs or palm leaflet pieces on the agar surface. Cultures were incubated at 25 ℃ under black light.

2.3. Fungal isolation

Leaflets and segments were first examined with a stereomicroscope for the presence of spore-producing structures. If no signs of sporulation were seen the specimens were incubated for 1–3 weeks in a moist chamber and examined daily with a stereomicroscope for signs of sporulation. When possible, isolations were made by direct transfer of conidia or ascospores onto CPDA. Isolations were also made directly from lesions after sterilization of pieces of tissue 1–2 mm^2 in 5% sodium hypochlorite for 1 min. These were then plated onto CPDA. Fungi were subcultured onto 1/2 PDA and single spore isolates established when possible.

2.4. Morphological observation and characterization

Microscopic structures were mounted in 100% lactic acid and examined by DIC microscopy. Observations on micromorphological features were made with Leica MZ9.5 and Leica DMR microscopes and digital images were recorded with Leica DFC300 and Leica DFC320 cameras, respectively. Measurements were made with the measurement module of the Leica IM500 Image Management System. Mean, SD and 95% confidence intervals were calculated from measurements of 50 structures, unless stated otherwise with n = total of measured structures. Measurements are given as minimum and maximum dimensions with mean and SD in parenthesis. Infrequent measurements are also given in parenthesis along with the minimum and maximum dimensions. Photoplates were prepared with Adobe Photoshop CS6.

2.5. Culture storage and preservation

Isolates were stored on 1/4 PDA slants about 2 cm in its widest part in 5 ml graduated microtubes and kept at 4 °C, and at room temperature after being covered with 2 ml of sterile mineral oil. Holotypes will be lodged at the herbarium of University of Aveiro (code AVE). Ex-type cultures will be deposited in the CBS collection at Westerdijk Institute, Utrecht, the Netherlands.

2.6. DNA extraction

Genomic DNA (gDNA) was extracted by a modified guanidium thiocyanate method. Isolates were grown on PDA in darkness at 20 °C until a suitable amount of mycelium growth was observed. The mycelium was then scraped off and collected in 2 ml microtubes with 100 µl of autoclaved glass microspheres and 250 µl of lysis buffer. The tubes were incubated on ice for 10 min, vortexed several times, incubated for 30 min at 65 ℃ and revortexed. If necessary, a pellet pestle was used to break the cell walls. Firstly 250 µl of GES reagent was added, mixed by inversion and the tubes kept on ice for 10 min. Then 250 µl of cold 10 M

ammonium acetate was added and the tubes returned to the ice bath for 10 min. To separate organic and aqueous phases, 1 ml of chloroform:isoamyl alcohol was added, mixed by vigorous agitation and then centrifuged (14 000 rpm, 20 min). The aqueous phase was transferred to a new 1.5 ml tube and the nucleic acids precipitated by adding an equal volume of cold absolute isopropanol and mixing by inversion. The tubes were centrifuged again (14 000 rpm, 20 min), the supernatant discarded and the pellets washed with 1 ml of cold 70% (v/v) ethanol. After a further centrifugation (14 000 rpm, 20 min), the supernatant was discarded and the pellets dried at room temperature with the tubes open in an inverted position. After ensuring that all the ethanol was removed, the pellets were resuspended in 100 µl of TE buffer and stored at 4 ℃.

Quality and quantity of the gDNA were evaluated by agarose gel electrophoresis. The gel was stained with 2.5 µg ml^{-1} ethidium bromide solution, visualized with an Alliance 4.7 UV transilluminator and the image recorded with Alliance software version 15.15. DNA concentrations were estimated using ImageJ software version 1.52a.

2.7. PCR amplification and sequencing

PCR reactions were carried out with Taq polymerase, nucleotides, primers, PCR-water (ultrapure DNase/RNase-free distilled water) and buffers supplied by Invitrogen (UK). PCR reaction mixtures and cycling conditions were optimized for each primer pair, with the addition of 5% DMSO or 0.01% BSA to improve the amplification of some difficult DNA templates. In some cases where amplification was not accomplished within a suitable range of concentrations adequate for sequencing, a second PCR was performed using as template 5 µl of the first PCR amplification. All amplification reactions were performed in a TGradient Thermocycler (Biometra, Germany). Amplified PCR products were purified and sequenced by Eurofins (Germany).

Primers ITS5 and NL-4 were used to amplify part of the cluster of rRNA genes, including the nuclear 5.8S rRNA gene and its flanking ITS1 and ITS2 regions, along with the first two domains of the large-subunit rRNA gene (ITS-D1/D2 rDNA region) (Table 2.1). The PCR reaction mixture consisted of 50–100 ng of gDNA, 1× PCR buffer, 50 pmol of each primer, 200 µM of each dNTP, 2 mM $MgCl_2$, 1 U Taq DNA polymerase and was made up to a total volume of 50 µl with PCR-water. The following cycling conditions were used: initial denaturation at 95 ℃ for 5 min, followed by 40 cycles of denaturation at 95 ℃ for 1 min, annealing at 52 ℃ for 30 s and elongation at 72 ℃ for 1.5 min, and a final elongation step at 72 ℃ for 10 min. The ITS region was sequenced only in the forward direction using the primer ITS5; the D1/D2 region (LSU) was sequenced only in the forward direction using the primers ITS5 and NL1 (5’ – GCATATCAATAAGCGGAGGAAAAG – 3’) (O’Donnell, 1993). If the forward sequencing did not resolve the sequence sufficiently, ITS region was also sequenced in the reverse direction using the primer ITS4 (5’ – TCCTCCGCTTATTGATATGC – 3’) (White *et al.*, 1990). Consensus sequences were produced with BioEdit version 7.0.5.3 (Hall, 1999).

The primers EF1-688F and EF1-1251R were used to amplify part of the translation elongation factor 1-alpha gene (TEF1), while the primers T1 and Bt2b were used to amplify part of the β-tubulin gene (TUB2) and the primers CAL-228F and CAL-737R were used to amplify part of the calmodulin gene (CAL) (Table 2.1). The PCR reaction mixture for each primer pair consisted of 50–100 ng of gDNA, 1× PCR buffer, 25 pmol of each primer, 200 µM of each dNTP, 3 mM $MgCl_2$, 1 U Taq DNA polymerase and was made up to a total volume of 25 µl with PCR-water. The following cycling conditions were used: initial denaturation at 95 ℃ for 5 min, followed by 40 cycles of denaturation at 95℃ for 30 s, annealing at 55 ℃, 52 ℃, 50 ℃ for 30 s (for TEF1, TUB2 and CAL, respectively) and elongation at 72 ℃ for 1 min, and a final elongation step at 72 ℃ for 10 min. All amplicons were sequenced in both directions, using the same primers as used for the DNA amplification, except for the TEF1 fragment, which was sequenced only in forward direction using the primer EF1-688F.

To assess PCR amplification, 5 µL of each PCR product was subjected to electrophoresis in a 0.8% (w/v) agarose (Invitrogen, UK) gel, with 0.5× TBE buffer (40 mM Tris, 45 mM boric acid, 1 mM EDTA, pH 8.3) and a constant voltage of 5.6 V cm^{-1} for 1 h. The molecular weight marker used was the 1 kb Plus DNA Ladder (Invitrogen, UK). The gel was stained and visualized as described above (subsection 2.6).

Table 2.1 Primer sets and corresponding amplification targets. Amplicon size represents the expected size in nucleotides for the amplified products considering the literature available, although small variations may be detected.

Gene/region	Primer	Sequence (5' – 3')	Amplicon size (nt*)	Reference
ITS-D1/D2	ITS5	GGAAGTAAAAGTCGTAACAAGG	1200	White et al., 1990
	NL4	GGTCCGTGTTTCAAGACGG		O'Donnell, 1993
TEF1	EF1-688F#	CGGTCACTTGATCTACAAGTGC	326	Alves *et al*., 2008
	EF1-1251R#	CCTCGAACTCACCAGTACCG		
CAL	CAL-228F	GAGTTCAAGGAGGCCTTCTCCC	500	Carbone and Kohn, 1999
	CAL-737R	CATCTTTCTGGCCATCATGG		
TUB2	T1	AACATGCGTGAGATTGTAAGT	500	O'Donnell and Cigelnik, 1997
	Bt2b	ACCCTCAGTGTAGTGACCCTTGGC		Glass and Donaldson, 1995

*nt, nucleotides.

#F, forward primer; R, reverse primer.

2.8. Sequence alignment and phylogenetic analyses

Sequences were edited with BioEdit version 7.0.5.3 (Hall, 1999) and aligned with ClustalX version 2.1 (Thompson *et al*., 1997) using the following parameters: pairwise alignment parameters (gap opening = 10, gap extension = 0.1) and multiple alignment parameters (gap opening = 10, gap extension = 0.2, DNA transition weigh = 0.5, delay divergent sequences = 25%). Additional sequences included in the alignments were obtained by subjecting the sequences for each gene region to megablast searches to identify closely related sequences from National Center for Biotechnology Information's (NCBI) GenBank sequence database (Benson *et al*., 2013) using Basic Local Alignment Search Tool (BLAST) (Altschul *et al*., 1990). Alignments were checked and manual adjustments were made where necessary using BioEdit version 7.0.5.3. Terminal regions with missing data in some of the isolates were excluded from the analysis.

Maximum Likelihood (ML) and Maximum Parsimony (MP) were used for phylogenetic inferences of single gene sequence alignments and the concatenated alignments. The individual gene trees were assessed for clade conflicts between the individual phylogenies. ML and MP inferences were implemented on the Cyberinfrastructure for Phylogenetic Research (CIPRES) Science Gateway (CSG) portal version 3.3 (Miller *et al*., 2010) using Randomized Axelerated Maximum Likelihood for High Performance Computing (RAxML-HPC2) version 8.2.12 (Stamatakis, 2014) and Phylogenetic Analysis Using Parsimony (PAUP) version 4.0a165 (Swofford, 2002), respectively. The resulting trees were plotted using TreeView version 1.6.6 (Page, 1996).

MP analyses were performed using the heuristic search option with 1000 random taxa additions and Tree Bisection and Reconnection (TBR) as the branch-swapping algorithm. All molecular characters were unordered and of equal weight, and alignment gaps were treated as missing data. Maxtrees were set to 1000 or 10000, branches of zero length were collapsed and all multiple, equally parsimonious trees were saved. Clade stability and robustness of the most parsimonious trees was assessed using bootstrap analysis with 1000 pseudoreplicates, each with 10 replicates of random stepwise addition of taxa (Felsenstein, 1985; Hillis and Bull, 1993). Descriptive tree statistical measures for parsimony calculated included tree length (TL), homoplasy index (HI), consistency index (CI) (Kluge and Farris, 1969), retention index (RI) and rescaled consistency index (RC) (Farris, 1989).

ML analyses were performed using a General Time Reversible (GTR) nucleotide substitution model (Tavaré, 1986) including a discrete gamma distribution (Yang, 1994) and estimation of proportion of invariable sites (Shoemaker and Fitch, 1989) (GTR+G+I) to accommodate variable rates across sites. Clade stability and robustness of the branches of the best-scoring ML tree were estimated by conducting a rapid bootstrap analysis with iterations halted automatically by RAxML.

Phylogenetic analyses were done on a matrix of concatenated ITS, TEF1, TUB2 and CAL sequences of representative species in *Diaporthe*. These were retrieved from GenBank according to the BLAST result for ITS locus and to the recent available literature on *Diaporthe*, such as Gomes *et al*. (2013), Gao *et al*. (2017),

Dissanayake *et al*. (2017b) and Marin-Felix *et al*. (2019b). Sequences obtained from GenBank are listed by their accession numbers, while newly generated sequences are listed by their isolate number and can be accessed in Supplementary Table A.10. The newly generated sequences will be deposited in GenBank. Sequences of *Diaporthe toxica* PM Will., Highet, W Gams & Sivasith were used as the outgroup taxon.

3. Results

3.1. Phylogenetic analyses

ITS, TUB2, TEF1 and CAL sequences of 64 strains of *Diaporthe* species, either sequenced in this study or retrieved from GenBank, were included in the phylogenetic analysis. The concatenated ITS, TUB2, TEF1 and CAL alignment of 63 ingroup and 1 outgroup taxa comprised 2248 characters including alignment gaps. Of the 2248 characters, 1229 were constant and 141 variable characters were parsimony-uninformative. MP analysis of the remaining 878 parsimony-informative characters resulted in 2496 equally parsimonious trees of 2546 steps with a low level of homoplasy as indicated by a CI of 0.616, a RI of 0.899, a HI of 0.384 and a RC of 0.554. The topology of the trees differed from one another only in the position of the isolates within terminal groupings, particularly within the clade that includes the species *D. phyllanthicola, D. loropetali* and *D. pseudophoenicicola*. Trees resulting from maximum parsimony and maximum likelihood analyses had similar topologies, except for the phylogenetic relationships between the same species in the previous referred clade. The ML tree is shown in Figure 3.14 with bootstrap support above the branches. It is to be noted that the phylogenetic position of *D. ceratozamiae* also differed in the two analyses. While in the MP tree it is more closely related to *D. arecae*, in the ML tree it is more closely related to *D. phyllanthicola*. In both phylogenies, however, this was not supported by a significant bootstrap value.

The seven isolates obtained in this study clustered in three different clades within *Diaporthe*, which are referred to as clade I, clade II and clade III (Figure 3.14). Only clades I and II received high bootstrap support (≥ 70%). Among these seven isolates, six corresponded to known *Diaporthe* species, namely *D. pyracanthae*, in clade I, *D. foeniculina*, in clade II, and *D. pseudophoenicicola*, in clade III. One isolate represented a previously undescribed species also included in clade III and closely related to *D. ceratozamiae*, *D. phyllanthicola* and *D. loropetali*. Herein this isolate is described as *D. chamaeropsicola*. Nevertheless, the phylogenetic position of taxa within clade III, including *D. chamaeropsicola*, generally received low bootstrap support (< 50%). Nevertheless, *Diaporthe chamaeropsicola* differs from *D. ceratozamiae*, *D. phyllanthicola* and *D. loropetali* in 13, 9 and 13, respectively, nucleotide positions in the ITS locus (Supplementary Table A.8).

3.2. Taxonomy

Based on morphological characteristics as well as DNA phylogeny, one of the isolates of *Diaporthe* collected from foliar lesions of palms was distinct from the previously known *Diaporthe* species and is described here as a new species. A description for this new species is provided below.

Diaporthe chamaeropsicola DRS Pereira & AJL Phillips sp. nov. (Figure 3.13)
MycoBank: MBXXXX

Etymology: named after the host genus from which it was collected, *Chamaerops humilis*.

Sexual morph: unknown. Asexual morph: *Conidiomata* pycnidial, solitary, occasionally aggregated, subglobose, dark-brown to black, thick-walled, up to 4 mm diam, covered with hyphal outgrows, superficial, lacking an ostiole, dehiscent by irregular fissures on pycnidial wall, exuding a creamy mucoid mass of conidia. *Pycnidial wall* pseudoparenchymatous of dark-brown *textura angularis*, cells thick-walled in outer layers, becoming thin-walled and hyaline towards the inner layers. *Conidiophores* reduced to conidiogenous cells. *Conidiogenous cells* straight, hyaline, smooth- and thin-walled, cylindrical, occasionally ampulliform, tapering towards the apex, aseptate or 1–3-septate, unbranched or branched, collarette up to 1 µm long, lining

the entire cavity, often intermingled with paraphyses, variable in length, dimorphic, *short conidiogenous cells*, 4.9–19.4 × 0.9–2.6 µm (mean ± SD = 13.66 ± 3.68 × 1.75 ± 0.39 µm), *long conidiogenous cells*, 15.2–49.2 × 1.1–2.7 µm (mean ± SD = 29.54 ± 7.28 × 1.75 ± 0.36 µm), enteroblastic proliferating at the same level giving rise to periclinal thickenings, occasionally enteroblastic proliferating percurrently giving rise to 1–2 annellations. *Paraphyses* straight, flexuous, hyaline, smooth- and thin-walled, cylindrical, tapering towards the apex, with 1–2–(3) basal septa, unbranched or branched below, often one of the branches later functioning as a conidiogenous cell, extending above conidiogenous cells, 26.6–78.8 µm (mean ± SD = 53.57 ± 12.72 µm) long. *Alpha conidia* cylindrical to ellipsoidal, mostly with rounded apex and obtuse to slightly truncate base, hyaline, smooth- and thin-walled, aseptate, biguttulate, with a conspicuous guttule at each end, occasionally with several minute scattered guttules, straight to slightly curved, 5.6–9.4 × 1.7–3.0 µm (mean ± SD = 7.53 ± 0.89 × 2.31 ± 0.30 µm); mean ± SD conidium length/width ratio = 3.33 ± 0.73. *Beta* and *gamma conidia* not seen.

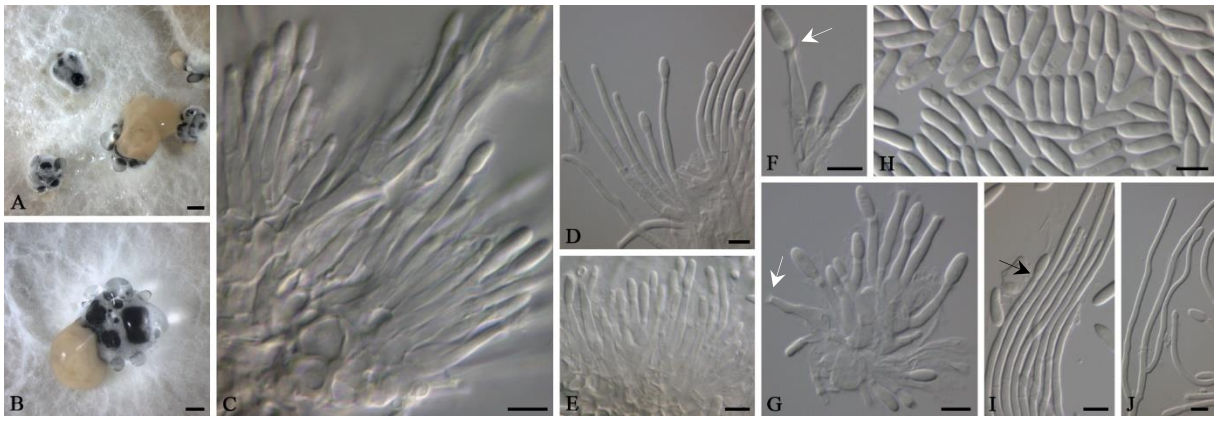

Figure 3.13 ***Diaporthe chamaeropsicola*** **(ex-type CDP 460/01)**. **A**, **B**. Conidiomata formed on 1/2 PDA. Conidia are oozing in creamy mucoid masses. **C**, **D**. Long conidiogenous cells. **E** – **G**. Short conidiogenous cells (white arrows point collarette) **H**. Conidia. **I**, **J**. Paraphyses (black arrow points a branch that are functioning as conidiogenous cell). Scale bars: A, B = 1 mm, C – J = 5 µm.

Culture characteristics: colonies on PDA, rapid growth, 60 mm diam after 7 d (n = 3). *Surface* flat, sparse aerial mycelium, often growing with concentric zones, with filiform margin, circular shape, pearl white to dirty white, opaque to slightly translucent. *Reverse* luteous, pale brown towards the centre. No diffusible pigment. Conidiomata black, scattered over the surface of the colony.

Material examined: Portugal, Lisbon, Parque das Nações, Jardins da Água, near Oceanário de Lisboa, on foliar lesions of segments of *Chamaerops humilis* (*Arecaceae*), 16 October 2018, Diogo RS Pereira (specimen HDP 034, holotype a dried culture of CDP 460, ex-type culture CDP 460/01, ITS sequence SDP 460/01, TUB2 sequence SDP 460/02, TEF1 sequence SDP 460/03, CAL sequence SDP 460/04).

Distribution: Lisbon, Portugal.

Notes: *Diaporthe chamaeropsicola* was found associated with foliar lesions of *Chamaerops humilis*, but pathogenicity has not been tested. The phylogenetic position of *D. chamaeropsicola* among accepted *Diaporthe* species is still not clearly resolved. Nevertheless, this species is phylogenetically related but distinct from *D. ceratozamiae*, *D. phyllanthicola* and *D. loropetali* (Figure 3.14). *Diaporthe chamaeropsicola* is morphologically similar to *D. ceratozamiae* (Figure 3.13), producing globose pycnidia, whose internal cavity is lined with cylindrical conidiogenous cells intermingled with long cylindrical, septate and branched paraphyses (Crous *et al*., 2011). However, *D. chamaeropsicola* has larger conidiomata than *D. ceratozamiae* (up to 4 mm diam vs. 300 µm diam), lacks conidiophores, and alpha conidia have a different shape (cylindrical/ellipsoidal vs. fusiform). In addition, they differ in 13 nucleotide positions in the ITS locus (Supplementary Table 3.8). *Diaporthe chamaeropsicola* differs from *D. phyllanthicola* and *D. loropetali* in

9 and 13, respectively, nucleotide positions in the ITS locus (Supplementary Table 3.8). No TUB2, TEF1 and CAL sequences are yet available for *D. ceratozamiae*, *D. phyllanthicola* or *D. loropetali*.

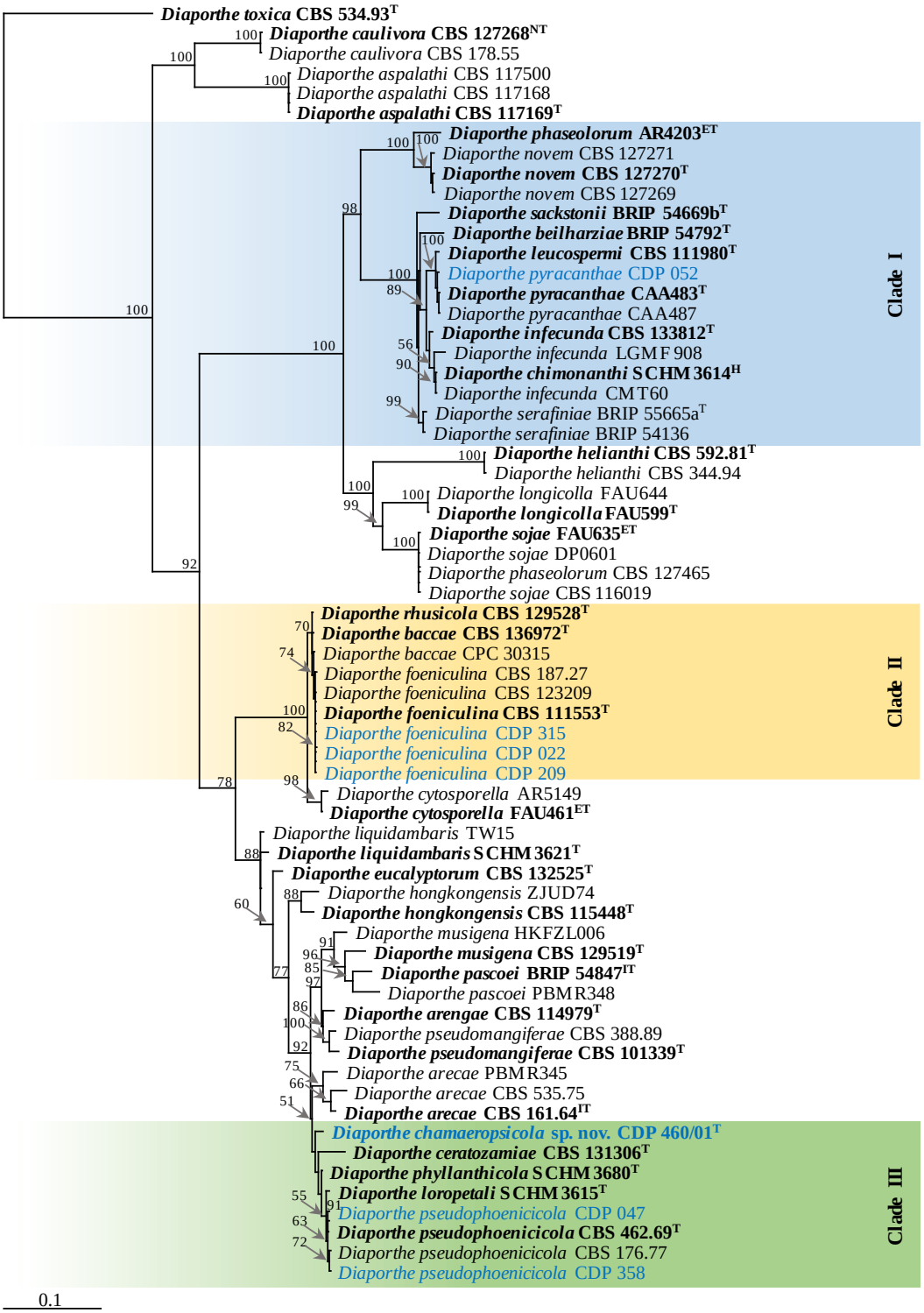

Figure 3.14 Phylogenetic position of *Diaporthe* isolates. ML tree generate by RAxML with GTR+G+I nucleotide substitution model using the combined four loci ITS-TEF1-TUB-CAL. ML bootstrap support values (> 50%) are shown above the branches. The isolates from this study are listed in blue. The scale bar represents the expected number of nucleotide changes per site. *Diaporthe toxica* (CBS 534.93) was included as outgroup. Ex-type (T)/ex-epitype (ET)/ex-isotype (IT)/ex-neotype (NT) cultures are marked in bold.

New host records of *Diaporthe foeniculina*, *D. pseudophoenicicola* and *D. pyracanthae* associated with foliar lesions of palms are also reported, along with a new insight into the distribution of *D. pseudophoenicicola*.

Diaporthe pseudophoenicicola RR Gomes, C Glienke & Crous, Persoonia 31: 30, 2013.

Type: Spain, Mallorca, Can Pastilla, dead tops of green leaves on *Phoenix dactylifera* (*Arecaceae*), 27 May 1969, HA van der Aa (holotype CBS H-21106, culture ex-type CBS 462.69).

Sexual morph not reported. See Gomes *et al*. (2013) for illustrations and descriptions of asexual morph.

Material examined: Portugal, Lisbon, Parque das Nações, Jardins da Água, Pomar do Mediterrâneo, on foliar lesions of segments of *Chamaerops humilis* (*Arecaceae*), 16 October 2018, Diogo RS Pereira (specimen HDP 039/02, living culture CDP 047, ITS sequence SDP 047/01, TUB2 sequence SDP 047/02, TEF1 sequence SDP 047/03, CAL sequence SDP 047/04); Portugal, Lisbon, Parque das Nações, on foliar lesions of leaflets of *Phoenix dactylifera* (*Arecaceae*), 16 October 2018, Diogo RS Pereira (specimen HDP 044/01, living culture CDP 358, ITS sequence SDP 358/01, TUB2 sequence SDP 358/02, TEF1 sequence SDP 358/03, CAL sequence SDP 358/04).

Distribution: China (Gao *et al*., 2017), Iraq (Shalt El Arab), Spain (Mallorca) (Gomes *et al*., 2013), Portugal (Lisbon) (present study).

Hosts: *Chamaerops humilis* (present study), *Mangifera indica*, *P. canariensis* (Gao *et al*., 2007), *Phoenix dactylifera* (Gomes *et al*., 2013; present study).

Notes: Two isolates of *D. pseudophoenicicola* were recorded from foliar lesions of palms, but pathogenicity has not been tested. This is the first time this *Diaporthe* species is reported from Portugal. One of the isolates was recorded from *C. humilis* and represents a new host record. The other isolate was recorded from *P. canariensis* and was already reported in the same host in collections from China (Gao *et al*., 2007). The present study confirms that *D. pseudophoenicicola* has a wide host range.

Diaporthe foeniculina (Sacc.) Udayanga & Castl., Persoonia 32: 95, 2014.

Type: Portugal, Madeira, Serra da Água, at base of 2-yr-old stem of *Foeniculum vulgare* (*Apiaceae*), August 2001, AJL Phillips (epitype LISE 94791, culture ex-type CBS 111553 = DP0391)

Sexual morph and asexual morph have been reported. See Phillips (2003) and Udayanga *et al*. (2014a) for illustrations and descriptions.

Material examined: Portugal, Lisbon, Parque das Nações, Jardins Garcia d'Orta, Talhão do Coloane, on foliar lesions of segments of *Trachycarpus fortunei* (*Arecaceae*), 5 October 2018, Diogo RS Pereira (specimen HDP 013/02, living culture CDP 022, ITS sequence SDP 022/01, TUB2 sequence SDP 022/02, TEF1 sequence SDP 022/03); Portugal, Lisbon, Marvila, Ferreira de Castro Street, near Caso dos Direitos Sociais, on foliar lesions of segments of *Chamaerops humilis* (*Arecaceae*), 13 October 2018, Diogo RS Pereira (specimen HDP 025/02, living culture CDP 209, ITS sequence SDP 209/01, TUB2 sequence SDP 209/02, TEF1 sequence SDP 209/03); Portugal, Lisbon, Parque das Nações, Jardins da Água, near Oceanário de Lisboa, on foliar lesions of segments of *Chamaerops humilis* (*Arecaceae*), 16 October 2018, Diogo RS Pereira (specimen HDP 035/02, living culture CDP 315, ITS sequence SDP 315/01, TUB2 sequence SDP 315/02, TEF1 sequence SDP 315/03).

Distribution: Argentina, Australia, Europe (Greece, Portugal, Spain, Italy), New Zealand, South Africa, USA (California) (Udayanga *et al*., 2014a; Lawrence *et al*., 2015; Annesi *et al*., 2016; Guarnaccia *et al*., 2016; present study).

Hosts: *Acacia* spp., *Acer* spp., *Actinidia deliciosa*, *Aspalathus linearis*, *Bougainvillea spectabilis*, *Camellia sinensis*, *Castanea* spp., *Chamaerops humilis*, *Citrus limon*, *C. limonia*, *Crataegus* spp., *Diospyros* spp., *Foeniculum vulgare*, *Fuchsia* spp., *Hydrangea* spp., *Juglans* spp., *Malus* spp., *Olea* spp., *Persea americana*, *Prunus* spp., *Pyrus* spp., *Quercus* spp., *Rhus* spp., *Ribes* spp., *Salix* sp., *Trachycarpus fortunei*, *Vitis vinifera*, *Wisteria sinensis* (Udayanga *et al*., 2014a; Lawrence *et al*., 2015; Annesi *et al*., 2016; Guarnaccia *et al*., 2016; present study).

Notes: Three isolates of *D. foeniculina* were recorded from foliar lesions of palms, but pathogenicity has not been tested. This is the first time this *Diaporthe* species is reported from *Arecaceae*, representing a new host record. Two of the isolates were recorded from *C. humilis*, another isolate was recorded from *Trachycarpus fortunei*. Thus, the present study gives a new insight into the wide host range already reported for *D. foeniculina*.

Diaporthe pyracanthae L Santos & A Alves, Mycosphere 8: 493, 2017.

Type: Portugal, Aveiro, from branch canker of *Pyracantha coccinea* (*Rosaceae*), March 2012, A Alves, (holotype LISE 96313, culture ex-type CBS142384 = CAA483).

Sexual morph not reported. See Santos *et al*. (2017) for illustrations and descriptions of asexual morph.

Material examined: Portugal, Lisbon, Parque das Nações, Jardins Garcia d'Orta, Talhão do Coloane, on foliar diseases of segments of *Chamaerops humilis* (*Arecaceae*), 16 October 2018, Diogo RS Pereira (specimen HDP 039/02, living culture CDP 052, ITS sequence SDP 052/01, TUB2 sequence SDP 052/02, TEF1 sequence SDP 052/03, CAL sequence SDP 052/04).

Distribution: Portugal (Aveiro, Lisbon) (Santos *et al*., 2017; present study).

Hosts: *Pyracantha coccinea* (Santos *et al*., 2017), *Chamaerops humilis* (present study).

Notes: One isolate of *D. pyracanthae* was recorded from foliar lesions of palms, but pathogenicity has not been tested. This is the first time this *Diaporthe* species is reported from *Arecaceae*, namely *Chamaerops humilis*, representing a new host record.

Review of *Diaporthe* names reported from *Arecaceae*

A search of the US National Fungus Collections Fungus-Host Database (Farr and Rossman, 2019) revealed 31 species of *Diaporthe*/*Phomopsis* associated with hosts in the family *Arecaceae*. These names were verified against MycoBank and Index Fungorum databases (Robert *et al*., 2005; Index Fungorum Partnership, 2009) as well as the available literature, including the most recent overviews of the taxonomy of the genus *Diaporthe* (e.g. Gomes *et al*., 2013; Gao *et al*., 2017; Dissanayake *et al*., 2017b; Marin-Felix *et al*., 2019b), which reduced the number to five *Diaporthe* species. Table 3.7 lists all current accepted names of *Diaporthe* species associated with *Arecaceae*, their respective hosts and countries from which they were recorded. *Diaporthe arctii* was described from *Trachycarpus fortunei* by Taylor and Hyde (2003) in Switzerland and the United Kingdom based solely on morphology. Since morphology is of limited value in defining species in *Diaporthe* (Hyde *et al*., 2011), the valid name for these isolates cannot be confirmed and thus the report was excluded from the list of species recorded on *Arecaceae* (Table 3.7). *Diaporthe eres* was recorded from diseased leaves of *Rhapis subtilis* by Gao *et al*. (2016). Nevertheless, Gao *et al*. (2006)

regarded its phylogenetic position as a species complex, since many of the isolates evaluated revealed ambiguous clades with short branches and moderate bootstrap support. Thus, the correct name for the species recorded on *R. subtilis* needs to be clarified. Udayanga *et al*. (2012a,b) recorded another *Diaporthe* species from *Rhapis* sp. in Thailand. Although this species is supported with molecular data, no morphological information was included, the species remained unnamed and was simply regarded as *Diaporthe* sp. Subsequently, this species was also disregarded from the present listing of *Diaporthe* names reported from *Arecaceae*.

Table 3.7 List of accepted *Diaporthe* names associated with *Arecaceae*. The *Diaporthe* species considered were only those described with both morphological and molecular data. Currently valid *Diaporthe* names that were described on palms based solely on morphological data were disregarded.

Species	Host	Country	Reference
Diaporthe arecae	*Areca catechu*	India	Gomes *et al*., 2013
Diaporthe arengae	*Arenga engleri*	China	Gomes *et al*., 2013
Diaporthe chamaeropis	*Chamaerops humilis*	Greece	Gomes *et al*., 2013
Diaporthe chamaeropsicola sp. nov.	*Chamaerops humilis*	Portugal	Present study
Diaporthe eres	*Rhapis subtilis*	China	Gao *et al*., 2016
Diaporthe foeniculina	*Chamaerops humilis*	Portugal	Present study
	Trachycarpus fortunei	Portugal	Present study
Diaporthe pseudophoenicicola	*Chamaerops humilis*	Portugal	Present study
	Phoenix canariensis	China	Gao *et al*., 2017
	Phoenix dactylifera	Spain	Gomes *et al*., 2013
	Phoenix dactylifera	Portugal	Present study
Diaporthe pyracanthae	*Chamaerops humilis*	Portugal	Present study

4. Discussion

In the present study four *Diaporthe* species were identified from diseased foliage of *Arecaceae* hosts. Of these, one was introduced as a new species, *D. chamaeropsicola*. Phylogenetically it forms a distinct lineage sister to *D. ceratozamiae*. Morphologically these two species are similar but the larger pycnidia, lack of conidiophores and cylindrical/ellipsoidal conidia differentiate it from *D. ceratozamiae*, which produces fusiform conidia in smaller pycnidia lined with aggregated, cylindrical conidiophores (Crous *et al*., 2011). Furthermore, a new insight into *Diaporthe* species associated with *Arecaceae* is present, with three new host reports for *D. foeniculina*, *D. pseudophoenicicola* and *D. pyracanthae*.

The phylogenetic position of *D. chamaeropsicola* is not fully resolved because of the low bootstrap support for this clade (Clade III in Figure 3.14). This lack of support is most likely because no sequences for TUB2, TEF1 and CAL are available for the other species in this clade. Nevertheless, from both ML and MP analyses, it is clear that this taxon represents a separate linage within the *D. pseudophoenicicola* clade. Furthermore, most of the base pairs differences found in *D. chamaeropsicola* were unique in this clade, which confirms the novelty. Despite the phylogenetic uncertainty, *D. chamaeropsicola* and *D. ceratozamiae* are morphologically similar tending to confirm that they form sister lineages. *Diaporthe phyllanthicola* and *D. loropetali* are also closely related to *D. chamaeropsicola* but also with low bootstrap support.

The phylogenetic uncertainty of *D. chamaeropsicola* due to lack of DNA sequence data of its nearest neighbours highlights the problems associated with applying the phylogenetic species concept to *Diaporthe*. This should be based on appropriate DNA barcodes that can give phylogenetic support to the clades and help to resolve species complexes. For example, Udayanga *et al.* (2014b) addressed this issue while assessing the species delimitation in the *D. eres* complex using a phylogenetic approach with multi-loci and clearly

resolved nine distinct phylogenetic species. Although revised several times (Gomes *et al*., 2013; Gao *et al*., 2017; Dissanayake *et al*., 2017b; Marin-Felix *et al*., 2019b), the taxonomy of *Diaporthe* continues to be confused and the genus urgently needs to be reassessed.

Case study II. *Morinia trachycarpae* sp. nov. and *Morinia phoenicicola* sp. nov., two new *Sporocadaceae* species from palms in Portugal

1. Introduction

The genus *Morinia* Berl. & Bres., based on *Morinia pestalozzioides* Berl. & Bres. from dried stems of *Artemisia camphorata*, was introduced by Berlese and Bresadola (1889) to accommodate species with appendage-bearing, muriform conidia developing in acervular conidiomata. In a later collection on the same host, Passerini (1891) described the fungus as *Pestalozia artemisiae* Pass. and assigned it to a new subgenus, *Pestalozziana* Pass. Saccardo (1892a,b) synonymized *Pestalozziana* under *Morinia* and *P. artemisiae* under *M. pestalozzioides*. These synonymies were accepted by Guba (1961) and Nag Raj (1993). Nieuwland (1916) considered that the Linnaean angiosperm genus *Morina* rendered *Morinia* a homonym. He introduced a new name *Rinomia* Nieuwl. to which he transferred the epithet *pestalozzioides* as *Rinomia pestalozzioides* Nieuwl. However, *R. pestalozzioides* is currently considered as an obligate or homotypic synonym of *M. pestalozzioides* (Kirk *et al*., 2001).

Morinia was a monotypic genus until 2006, when Collado *et al*. (2006) isolated and described the new species *Morinia longiappendiculata* Collado & Platas from healthy living stems and leaves of *Calluna vulgaris*, *Santolina rosmarinifolia*, *Helichrysum stoechas* and *Thymus mastichina* based on both morphological and molecular data. Compared with *M. pestalozzioides*, *M. longiappendiculata* produces larger and more fusiform conidia, with larger basal and apical appendages, generated on filiform, instead of cylindrical, conidiogenous cells. In the same studies, the type species *M. pestalozzioides* was redescribed based on an authentic specimen (BPI 453814) that was designated as the lectotype, since no holotype was designated for this type species (Berlese and Bresadola, 1889; Guba, 1961; Nag Raj, 1993). Collado *et al*. (2006) also designated an epitype for *M. pestalozzioides* isolated from living stems of *Sedum sediforme*. The ITS sequence analyses by Collado *et al*. (2006) placed *Morinia* in a monophyletic clade together with *Bartalinia* and *Truncatella* species within the *Sporocadaceae* (as *Amphisphaeriaceae*), which includes other morphologically similar genera of coelomycetes, such as *Pestalotiopsis* and *Seiridium* (Jeewon *et al*., 2002; Hongsanan *et al*., 2017).

In recent years, multi-locus phylogenetic analyses aimed at basing the taxonomy of *Sporocadaceae* within a natural classification have revealed two new species of *Morinia* characterized by only transverse conidia, such as *Morinia acaciae* (Crous) F Liu, L Cai & Crous and *Morinia crini* F Liu, L Cai & Crous (Liu *et al*., 2019). Liu *et al*. (2019) also emended the generic circumscription of *Morinia*. *Zetiasplozna acaciae* Crous was described by Crous *et al*. (2014) from leaves of *Acacia melanoxylon* due to its general morphological resemblance to *Zetiasplozna thuemenii* (Speg.) Nag Raj (Nag Raj, 1993). However, it was later transferred to *Morinia* as *M. acaciae* due to the lack of any sequences of the generic type *Zetiasplozna unicola* (Berk. & MA Curtis) Nag Raj. In addition, *M. crini* was introduced as a new species on *Crinum bulbispermum*. Both species are morphologically similar and phylogenetically related to *M. pestalozzioides* and *M. longiappendiculata* (Liu *et al*., 2019).

Two interesting *Bartalinia*-like fungi were isolated from foliar lesions on palms in Lisbon, Portugal. These fungi fit well within the current concept of *Morinia* and can be distinguished from *Bartalinia* by characteristics of the centric basal appendage and axial and lateral appendages on the apical cell. Morphological examination and phylogenetic analyses showed that these two fungi differ from all other previously described species in *Morinia* (Berlese and Bresadola, 1889; Collado *et al*., 2006; Crous *et al*., 2014; Liu *et al*., 2019) and are therefore described here as *Morinia trachycarpae* DRP Pereira & AJL Phillips and *Morinia phoenicicola* DRP Pereira & AJL Phillips spp. nov.

2. Materials and Methods

All the methods used here were the same as those described in Case Study I except that only ITS and LSU loci were included in the phylogenetic analyses.

Phylogenetic analyses were done on a matrix of concatenated ITS and LSU sequences of representative genera and species in *Sporocadaceae*. These were retrieved from GenBank according to the BLAST result for each locus and to the recent available literature on *Sporocadaceae*, such as Hongsanan *et al*. (2017) and Liu *et al*. (2019). Sequences obtained from GenBank are listed by their accession numbers, while newly generated sequences are listed by their isolate number and can be accessed in Supplementary Table A.11 The newly generated sequences will be deposited in GenBank. Sequences of *Clypeosphaeria mamillana* (Fr.) Lambotte and *Lepteutypa sambuci* Jaklitsch & Voglmayr were used as the outgroup taxa.

3. Results

Four strains with the general morphological features of *Morinia* were isolated from foliar lesions of palms. ITS and/or LSU were sequenced and included in phylogenetic analyses to determine their relationship with known species.

3.1. Phylogenetic analyses

The ITS and LSU sequences of 32 strains of *Sporocadaceae* family, either sequenced in this study or retrieved from GenBank sequences database, were included in the phylogenetic analysis. The concatenated ITS and LSU alignment of 32 ingroup and 2 outgroup taxa comprised 1105 characters including alignment gaps. Of the 1105 characters, 853 were constant and 112 variable characters were parsimony-uninformative. MP analysis of the remaining 140 parsimony-informative characters resulted in 2994 equally parsimonious trees of 407 steps with a low level of homoplasy as indicated by a CI of 0.732, RI of 0.823, HI of 0.152 and RC of 0.602. Topology of the trees differed from one another only in the positions of the isolates within terminal groupings and between the clade of *Hymenopleella* and the remaining clades. Tree topologies resulting from maximum parsimony and maximum likelihood analyses were similar, except for the *Heterotruncatella* and *Truncatella* clade, and only the former is shown in Figure 3.15 with bootstrap support values above the branches.

Isolates obtained in this study clustered in the *Morinia* clade with high bootstrap support (> 90%) and were distinct from *Bartalinia* and *Truncatella* clades, as well as from other *Sporocadaceae* genera clades close to *Morinia*, such as *Heterotruncatella* and *Hymenopleella*. The isolates obtained from foliar lesions of palms formed two different sister groups. Two isolates (CDP 191 and CDP 192) formed a group sister to *M. acaciae* while CDP 097 and CDP 232 formed a group sister to *M. crini* within the *Morinia* clade with moderate bootstrap support (69% and 63%, respectively) (Figure 3.15). The clade composed of *M. acaciae* and CDP 191 and CDP 192 is closer to the clade composed of *M. longiappendiculata* and *M. pestalozzioides* than the clade composed of *M. crini* and CDP 097 and CDP 232 with moderate bootstrap support (75%).

Although the ML analysis resulted in a tree with similar topology to the MP tree, most of the internal nodes received low bootstrap support but in general the clades constituting the genera received high bootstrap support, with 89% for *Morinia* clade, where *M. trachycarpae* and *M. phoenicicola* clustered in groups sister to the previous known species of *Morinia* in a similar way as noted for the MP analysis. ML bootstrap values for the phylogenetic relationships between the isolates obtained in this study and *Morinia* species were as high or higher comparatively to the MP bootstrap values (74% for the group sister with CDP 097 and CDP 232 and 70% for the group sister with CDP 191 and CDP 192).

3.2. Taxonomy

Based on morphological characteristics as well as phylogenetic analysis, the fungi collected from foliar diseases of palms were considered to be distinct from all previously described *Morinia* species. Therefore, they are introduced as new and descriptions are provided below, along with an emendation of the genus description.

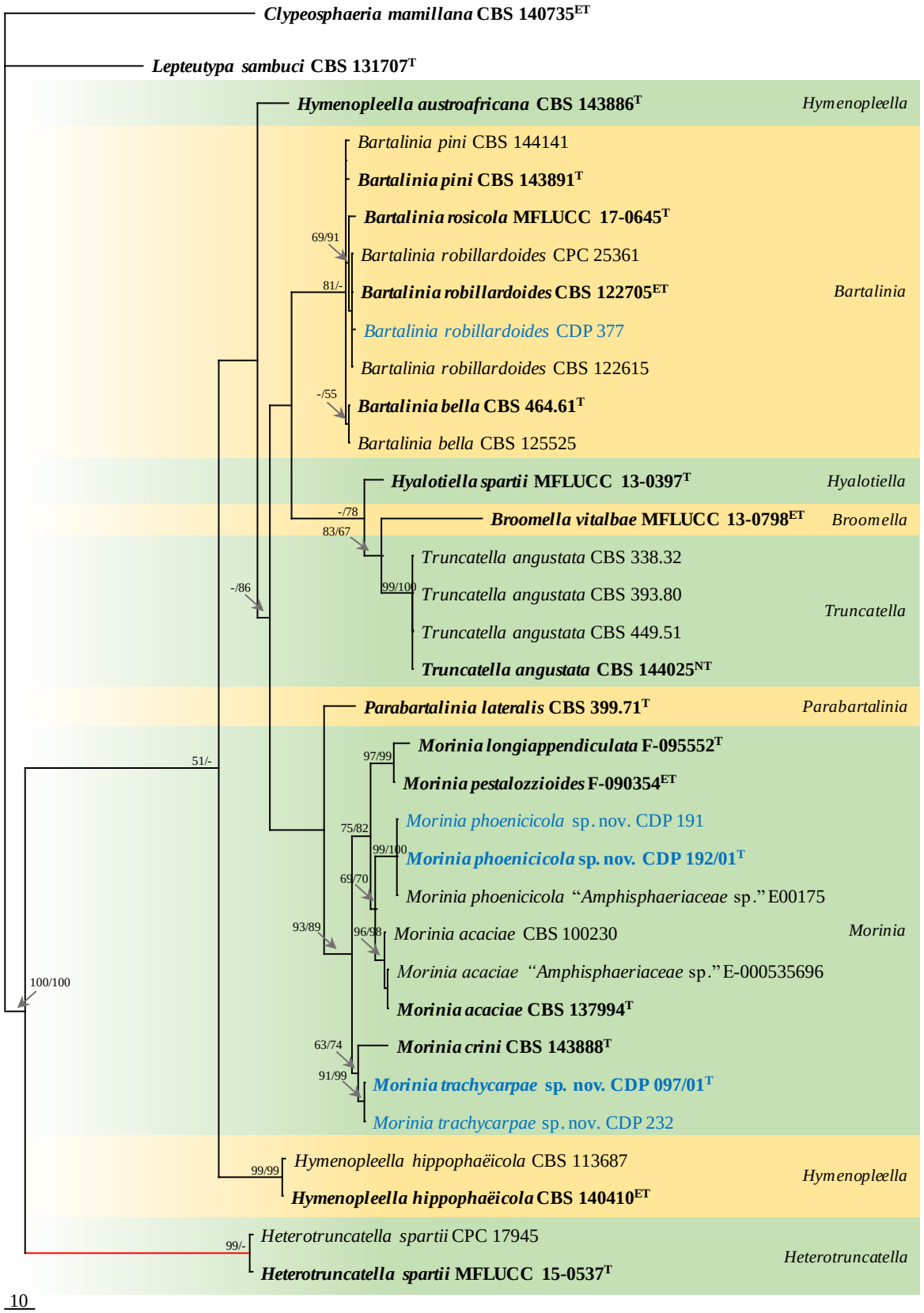

Figure 3.15 Phylogenetic position of *Morinia* isolates. One of 2994 equally parsimonious phylogenetic trees obtained from the combined ITS and LSU sequence alignment (TL = 407 steps, CI = 0.732, RI = 0.823, HI = 0.152 and RC = 0.602). Bootstrap support values (> 50%) for MP and ML analyses are shown above the branches as MP/ML. Isolates from this study are listed in blue. Branch marked in red is not present in the ML tree. The scale bar represents the expected number of nucleotide changes per site. *Clypeosphaeria mamillana* (CBS 140735) and *Lepteutypa sambuci* (CBS 131707) were included as outgroups. Ex-type (T)/ex-epitype (ET)/ex-neotype (NT) cultures are marked in bold.

Morinia Berl. & Bres., Annuario Società Alpinisti Tridentini 14: 82, 1889 [1887–1888], emend. DRS Pereira & AJL Phillips.

Type species: *Morinia pestalozzioides* Berl. & Bres., Annuario Società Alpinisti Tridentini 14: 82, 1889.

Description: Sexual morph: unknown. Asexual morph: *Conidiomata* acervular, stromatic, pycnidioid or pycnidial, superficial or semi-immersed, erumpent, scattered, solitary or aggregated, globose or subglobose, glabrous pale brown, brown or dark, ostiolate or non-ostiolate. *Stroma and wall* of brown *textura angularis*, cells globose, oblong to angular, thick-walled and brown in outer layers, becoming thin-walled and subhyaline to pale brown or hyaline towards the inner layers. *Conidiophores* arising from all around the cavity of conidioma from the innermost wall layer, aseptate and unbranched or septate and branched, or reduced to conidiogenous cells, hyaline, smooth- and thin-walled, invested in mucus or not. *Conidiogenous cells* ampulliform, cylindrical, subcylindrical or lageniform, hyaline or pale brown, smooth- and thin-walled. *Conidia* fusiform, ellipsoidal, subcylindrical or lunate, transversely septate, muriformly septate or not, straight to slightly curved, smooth or verruculose-walled, thin-walled, with or without constriction at the septa, median cells pale brown to brown or hyaline, end cells hyaline or pale brown, apical cell hemispherical or wedge-shaped, bearing appendages at both ends; appendages attenuated or not, unbranched, aseptate, tubular, filiform or cylindrical, flexuous or not; appendages on apical cell several, inserted at different loci; basal appendage single, rarely two or more, centric or excentric.

Distribution: France, Germany, Italy, New Zealand, Poland, Portugal and Spain.

Hosts: *Acacia melanoxylon*, *Artemisia camphorata*, *Calluna vulgaris*, *Crinum bulbispermum*, *Helichrysum stoechas*, *Phoenix canariensis*, *Phoenix dactylifera*, *Santolina rosmarinifolia*, *Trachycarpus fortunei* and *Thymus mastichina*.

Notes: *Morinia* is characterized by muriform or transverse and appendage-bearing conidia in acervular, stromatic or pycnidioid conidiomata (Liu *et al*., 2019) and previously included four asexual species, *M. pestalozzioides*, *M. longiappendiculata*, *M. acaciae* and *M. crini*. In this study, two species characterized by only transverse conidia are incorporated in this genus, one of which producing enclosed pycnidia in culture and for this reason the generic description is emended.

Morinia trachycarpae DRS Pereira & AJL Phillips sp. nov. (Figure 3.16)
MycoBank: MBXXXX

Etymology: named after the host genus from which it was collected, *Trachycarpus fortunei*.

Sexual morph: unknown. Asexual morph: *Conidiomata* pycnidial, non-stromatic, solitary, occasionally aggregated, globose to subglobose, glabrous, often covered with hyphal outgrows, superficial, lacking an ostiole, light-brown to dark-brown, thin-walled, variable in size, up to 300 µm diam. *Pycnidial wall* of brown *textura angularis*, cells thick-walled and brown in outer layers, becoming thin-walled and paler or hyaline towards the inner layers. *Conidiophores* arising all around the cavity of conidioma from the innermost wall layer, with a supporting narrow cell, often reduced to conidiogenous cells, hyaline, smooth- and thin-walled, cylindrical to subcylindrical or doliiform, variable in size, 2.5–9.1 × 2.0–7.7 µm (mean ± SD = 5.40 ± 1.67 × 3.75 ± 1.44 µm). *Conidiogenous cells* hyaline, smooth- and thin-walled, mostly cylindrical, subcylindrical, sometimes lageniform, rarely 1-septated, with a small branch below the septum, variable in size, 2.4–14.2 × 1.4–9.2 µm (mean ± SD = 8.06 ± 2.66 × 2.44 ± 1.21 µm), enteroblastic proliferating at the same level giving rise to periclinal thickenings or proliferating percurrently giving rise to 2–3 annellations near the apex. *Conidia* subcylindrical, sometimes lunate, widest in the upper region, straight to slightly curved, 4-septate,

septa often inconspicuous, not-constricted at the septa, often becoming slightly to highly constricted with age, with minute to big, pigmented, light-brown to pale guttules, often located near the septa, bearing appendages on both ends, 18.5–25.9 × 3.4–5.6 µm (mean ± SD = 22.60 ± 1.57 × 4.47 ± 0.52 µm); basal cell hyaline, smooth- and thin-walled, obconical with truncate, rarely obtuse, base, 3.6–5.6 × 2.1–3.8 µm (mean ± SD = 4.57 ± 0.53 × 2.85 ± 0.33 µm); three median cells hyaline, rarely subhyaline, pale-brown or goldish, smooth- and thin-walled, subcylindrical, guttulate, together 11.3–16.4 × 9.8–16.4 µm (mean ± SD = 14.35 ± 1.14 × 12.57 ± 1.49 µm), ± equal in the first two median cells from apex, third cell longer, each 3.8–5.5 × 3.3–5.5 µm (mean ± SD = 4.78 ± 0.38 × 4.19 ± 0.50 µm); apical cell hyaline, rarely subhyaline to pale-brown, smooth- and thin-walled, wedge-shaped, 2.4–5.4 × 2.4–4.4 µm (mean ± SD = 3.68 ± 0.65 × 3.38 ± 0.39 µm); basal and cells usually devoid of contents; apical appendages, 2–3, rarely 1, arising laterally and axially, hyaline, smooth- and thin-walled, unbranched, attenuated, filiform, tubular, flexuous, 10.3–24.3 µm (mean ± SD = 16.56 ± 3.10 µm) long; basal appendage, single, centric, rarely uncentred, hyaline, smooth- and thin-walled, unbranched, attenuated, filiform, tubular, erect, 3.0–18.1 µm (mean ± SD = 9.39 ± 3.16 µm) long; mean ± sd conidium length/width ratio = 5.11 ± 0.62.

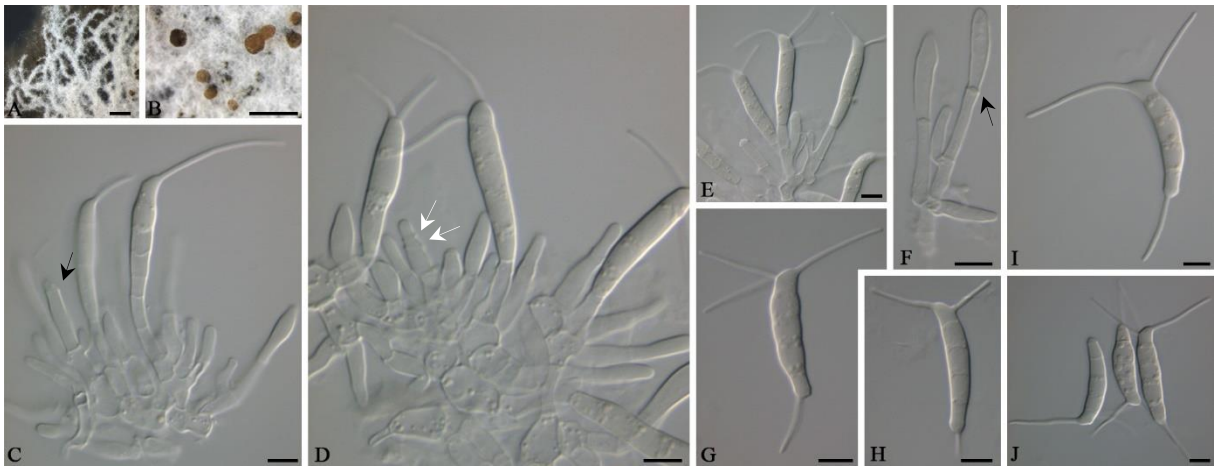

Figure 3.16 ***Morinia trachycarpae*** **(ex-type CDP 097/01)**. **A**, **B**. Growth appearance and conidiomata formed on 1/2 PDA. **C** – **F**. Conidiogenous cells with periclinal thickenings (black arrows) or annellations (white arrows). **G** – **J**. Conidia. Scale bars: A, B = 1 mm, C – J = 5 µm.

Culture characteristics: colonies on PDA, moderate rapid growth, 37 mm diam after 7 d (n = 3). *Surface* flat, glabrous to velvety, with filiform to entire margin, circular shape, dirty white to grey, successively pale luteous-straw to the margin, opaque. *Reverse* concolorous, peanut brown to buff, paler to the margin. No diffusible pigment. Conidiomata covered with mycelium growth and not visible through the surface of colony.

Material examined: Portugal, Lisbon, Parque das Nações, Jardins da Água, Pomar do Mediterrâneo, on foliar diseases of leaflets of *Trachycarpus fortunei* (*Arecaceae*), 16 October 2018, Diogo RS Pereira (specimen HDP 042, holotype a dried culture of CDP 097, ex-type culture CDP 097/01, ITS sequence SDP 097/01, LSU sequence SDP 097/02); Portugal, Lisbon, Alvalade, Campo Grande, on foliar diseases of leaflets of *Phoenix dactylifera* (*Arecaceae*), 5 October 2018, Diogo RS Pereira (specimen HDP 012, living culture CDP 232, ITS sequence SDP 232/01, LSU sequence SDP 232/02).

Distribution: Lisbon, Portugal.

Notes: *Morinia trachycarpae* was found associated with foliar diseases of *Trachycarpus fortunei* and *Phoenix dactylifera*, but pathogenicity has not been proved. Although *M. trachycarpae* is closely related to *M. crini* (Figure 3.15), it resembles *M. acaciae* in morphology (Figure 3.16, Table 3.8), producing subcylindrical,

widest in the upper region, transversely-septate conidia with one lateral and one axial appendages in an apical wedge-shaped cell (Crous *et al*., 2014). However, *M. trachycarpae* differs from *M. acaciae* in the length of

Table 3.8 Comparative morphological data on species of *Morinia*.

Characters	*Morinia pestalozzioides*[1]	*Morinia longiappendiculata*[2]	*Morinia acaciae*[3]	*Morinia crini*[4]	*Morinia trachycarpae*	*Morinia phoenicicola*
Conidia						
Shape	Fusiform, ellipsoid, pyriform	Fusiform, ellipsoid, pyriform	Subcylindrical, widest in the upper region	Cylindrical, subcylindrical, lunate	Subcylindrical, sometimes lunate, widest in the upper region	Fusiform to ellipsoidal, subcylindrical
Colour	End cells hyaline, median cells brown	End cells hyaline, median cells brown	Subhyaline, basal cell hyaline	End cells hyaline to pale brown, median cells pale brown	Hyaline	End cells hyaline, median cells pale brown
Size (µm)	20–25 × 6–8	25–31 × 9–11	(31–)33–37(–41) × 4(–5)	18–22 × 4–5	19–26 × 3–6	21–35 × 3–9
Transverse septa	(5–)6–7	5–6	4	4	4	3
Vertical septa	1–2	(1–)2(–3)	–	–	–	–
Oblique septa	1–2	(1–)2	–	–	–	–
Median cells length (µm)	15–17	17–20	–	10–15	11–16	10–18
No. of apical appendages	(2–)3	(2–)3	2	2	(1–)2–3	(2–)3(–4)
Apical appendages length (µm)	9–11	14–26	12–27	8–12	10–24	10–24
No. of basal appendages, position	0(–1–2), centric	1, centric or excentric	1, centric	1(–2), centric or excentric	1, centric	1(–2–4), centric
Basal appendages length (µm)	3	15–25	2–8	4–9	3–18	6–22
Conidiogenous cells						
Shape	Cylindrical	Filiform	Ampulliform	Mostly cylindrical, subcylindrical, sometimes lageniform	Subcylindrical, sometimes lageniform	Cylindrical to subcylindrical
Branching	Yes	Yes	No	No	Rarely	Rarely
Size (µm)	10–29 × 2–3	23–42 × 2–3	7–12 × 3–4	6–21 × 2–3	2–14 × 1–9	5–17 × 1–6
Septation	Yes	Yes	No	No	Rarely	Rarely
Colour	Hyaline	Hyaline	Hyaline	Hyaline	Hyaline	Hyaline, subhyaline to pale brown

[1]Description according to Berlese and Bresadola (1889) and Collado *et al*. (2006).
[2]Description according to Collado *et al*. (2006)
[3]Description according to Crous *et al*. (2014).
[4]Description according to Liu *et al*. (2019).

conidia (19–26 µm vs. 33–37 µm) and basal appendage (3–18 µm vs. 2–8 µm) and in the number of apical appendages (2–3 vs. 2). In addition, *M. trachycarpae* conidia are hyaline, while *M. acaciae* conidia are subhyaline with a basal hyaline cell. *M. trachycarpae* differs widely from *M. crini* in several micromorphological characteristics, such as the shape and the colour of conidia (subcylindrical hyaline vs. cylindrical hyaline to pale brown), the number of apical appendages (2–3 vs. 2) and the length of apical (10–24 µm vs. 8–12 µm) and basal appendages (3–18 µm vs. 4–9 µm), comprising in general much longer and wider conidia. *M. trachycarpae* also resembles *Zetiasplozna thuemenii* (on leaves and fruit of diverse hosts) (Nag Raj, 1993), except that it has relatively shorter but wider conidia (19–26 × 3–6 vs. 20–32 × 4–5) and a relatively smaller mean conidium length/width ratio (5.11 ± 0.62 vs. 5.7 ± 1). Two strains of *M. trachycarpae* were isolated from foliar lesions of palms, namely CDP 097/01 (ex-type) and CDP 232. They exhibited a similar colony morphology when cultured on 1/2 PDA and the nucleotide sequence similarity between them was 100% for ITS and 100% for LSU. No relevant variation in micromorphology was observed between these strains. *M. trachycarpae* and *M. crini* differ in several nucleotide positions in the following loci: ITS (10 nt) and LSU (6 nt) (Supplementary Table A.9).

Morinia phoenicicola DRS Pereira & AJL Phillips sp. nov. (Figure 3.17)
MycoBank: MBXXXX

Etymology: named after the host genus from which it was collected, *Phoenix canariensis*.

Sexual morph: unknown. Asexual morph: *Conidiomata* acervular, with a basal discoid stroma of *textura angularis* compose of globose, oblong to angular, subhyaline to pale-brown, thin-walled cells, globose to subglobose, solitary or aggregated, dark-brown to black, partially immersed to superficial, variable in size, up to 600 µm diam, filled with ochraceous, dark-brown to black globose mucoid mass of conidia. *Conidiophores* reduced to conidiogenous cells. *Conidiogenous cells* hyaline, subhyaline to pale brown, smooth- and thin-walled, cylindrical to subcylindrical, aseptate to 1-septate, with a small branch below the septum, variable in size, 4.8–16.6 × 1.4–5.5 µm (mean ± SD = 8.26 ± 2.74 × 2.90 ± 0.92 µm), enteroblastic proliferating at the same level giving rise to periclinal thickenings or proliferating percurrently giving rise to 1–3 annellations near the apex. *Conidia* fusiform to ellipsoidal, subcylindrical, straight to slightly curved, 3-septate, highly constricted at the septa, eguttulate, rarely with big, pigmented, light-brown guttules in median cells, bearing appendages on both ends, 20.7–34.9 × 3.2–9.0 µm (mean ± SD = 27.62 ± 2.79 × 6.69 ± 1.17 µm); basal cell hyaline, rarely subhyaline to pale brown, smooth- and thin-walled, obconical, with obtuse base, 2.4–7.3 × 2.3–7.0 µm (mean ± SD = 3.66 ± 0.74 × 3.39 ± 0.72 µm); two media cells pale brown, smooth and thin-walled, subcylindrical to doliiform, guttulate, together 9.6–17.6 × 6.0–17.9 µm (mean ± SD = 13.61 ± 1.79 × 12.82 ± 2.35 µm), first median cell from apex slightly longer and wider, each 4.8–8.8 × 3.0–8.9 µm (mean ± sd = 6.80 ± 0.89 × 6.41 ± 1.18 µm); apical cell hyaline, smooth- and thin-walled, conical, with obtuse apex, 2.6–4.6 × 2.2–3.9 µm (mean ± SD = 3.55 ± 0.43 × 3.15 ± 0.43 µm); apical appendages, 2–4, mostly 3, arising laterally and axially, hyaline, smooth- and thin-walled, attenuated or not-attenuated, filiform, tubular, flexuous, 10.2–23.9 µm (mean ± SD = 15.14 ± 2.66 µm) long; basal appendage wider, up to 2 µm wide, mostly single, occasionally 2–4, centric, hyaline, smooth- and thin-walled, not-attenuated, cylindrical, tubular, erect to flexuous, 6.1–22.2 µm (mean ± SD = 13.14 ± 3.80 µm) long; mean ± SD conidium length/width ratio = 4.22 ± 0.62.

Culture characteristics: colonies on PDA, moderate slow growth, 22 mm diam after 7 d (n = 3). *Surface* flat, velvety, wrinkled, with lobate to undulate margin, irregular shape, grey olivaceous to glaucous grey, opaque. *Reverse* concolorous, dark-brown to black. Presence of yellowish to deep-orangish diffusible pigment. Conidiomata black, gregarious to successively scattered to the margin.

Material examined: Portugal, Lisbon, Areeiro, Humberto da Cruz street, on foliar diseases of leaflets of *Phoenix canariensis* (*Arecaceae*), 6 October 2018, Diogo RS Pereira (specimen HDP 022/02, holotype a

dried culture of CDP 192, ex-type culture CDP 192/01, ITS sequence SDP 192/01; specimen HDP 022/01, living culture CDP 191, ITS sequence SDP 191/01).

Distribution: Lisbon, Portugal.

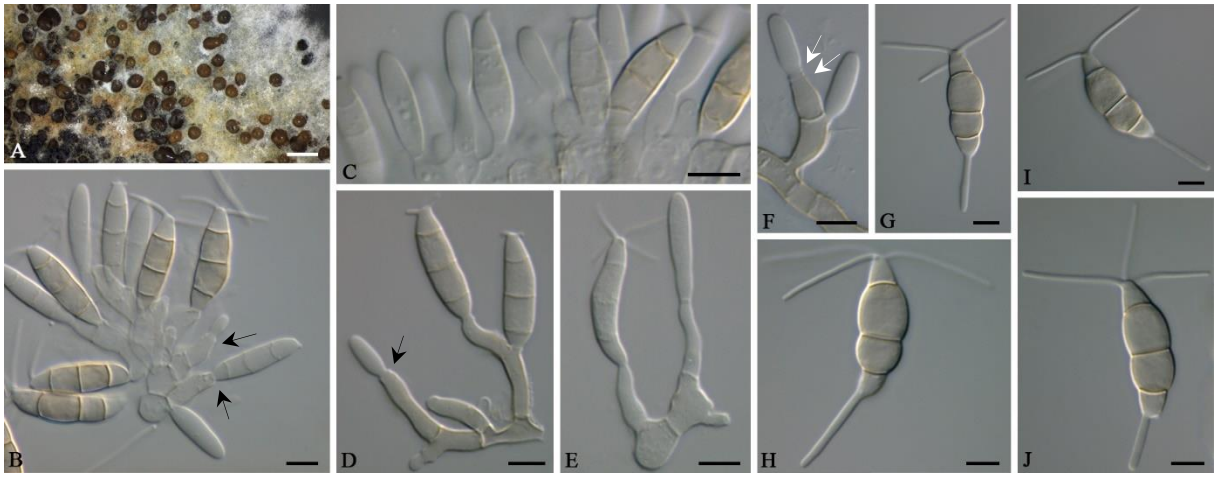

Figure 3.17 ***Morinia phoenicicola*** **(ex-type CDP 192/01)**. **A**. Conidiomata formed on 1/2 PDA. Conidia are oozing in dark brown to black mucoid masses. **B – F**. Conidiogenous cells with periclinal thickenings (black arrows) or annellations (white arrows). **G – J**. Conidia. Scale bars: A = 1 mm, B – J = 5 µm.

Notes: *Morinia phoenicicola* was found associated with foliar diseases of *Phoenix canariensis*, but pathogenicity has not been proved. Although *M. phoenicicola* is closely related to *M. acaciae* (Figure 3.15), it resembles *M. pestalozzioides* and *M. longiappendiculata* in morphology (Figure 3.17, Table 3.8), producing fusiform to ellipsoidal conidia with mostly 2 lateral and 1 axial apical appendages (Collado *et al*., 2006). However, *M. phoenicicola* differs from both *M. pestalozzioides* and *M. longiappendiculata* in the septation of conidia (only transversely septate vs. muriformly septate) and in the size of conidiogenous cells (5–17 × 1–6 vs. 10–29 × 2–3 and 23–42 × 2–3, respectively). Morphologically, *M. phoenicicola* is more similar to *M. longiappendiculata* than *M. pestalozzioides*. While *M. pestalozzioides* conidia mostly lack basal appendage, *M. phoenicicola* conidia mostly present a long basal appendage and are larger similar to what is found on *M. longiappendiculata* (20–25 × 6–8 µm for *M. pestalozzioides* and 25–31 × 9–11 and 21–35 × 3–9 for *M. longiappendiculata* and *M. phoenicicola*, respectively). *M. phoenicicola* differs from all the other *Morinia* species in having a smaller number of transverse septa (3 vs. 4 in *M. trachycarpae*, *M. crini* and *M. acaciae*, 5–6 in *M. longiappendiculata* and 6–7 in *M. pestalozzioides*). Two strains of *M. phoenicicola* were isolated from foliar lesions of palms, namely CDP 191 and CDP 192/01 (ex-type). They exhibited a minute degree of variation in colony morphology when cultured on 1/2 PDA and the nucleotide sequence similarity between them was 99.80% for ITS, which results from a single nucleotide position difference, i.e., an additional G in the beginning of ITS sequence in CDP 192/01. Colony morphology variation is expressed only in the fact that CDP 192/01 produced consistently a higher number of gregarious conidiomata through the surface of the colonies. No relevant variation in micromorphology was observed between these strains. *Morinia phoenicicola* and *M. acaciae* differ in 12 nucleotide positions in the ITS locus (Supplementary Table A.9). No LSU sequences for *M. phoenicicola* are yet available.

4. Discussion

In the present study, two new species are introduced in *Morinia*, namely *M. trachycarpae* and *M. phoenicicola*. Morphological characters distinguished them from each other and from all the other known species in the genus. In addition, phylogenetic analyses based on ITS and LSU sequences confirmed these novelties. These species are the first report of *Morinia* in Portugal and also the first time that *Morinia* species have been reported on palm trees (*Arecaceae*).

A survey of the literature suggests that *Morinia* is a rare genus previously known from a few specimens of angiosperms from Italy, Spain, France, Poland, Germany and New Zealand (Berlese and Bresadola, 1889; Collado *et al*., 2006; Adamska, 2007; Weber *et al*., 2007; Crous *et al*., 2014; Liu *et al*., 2019). In these reports, several families of flowering plants were shown to be hosts of *Morinia* species, such as *Amaryllidaceae*, *Asteraceae*, *Crassulaceae*, *Ericaceae*, *Lamiaceae* and *Fabaceae*. Most of these families are present in European temperate countries. However, the present study reports this genus from *Arecaceae*, a family of angiosperms typical of tropical and subtropical climates. This included three different palm trees species, namely *Phoenix canariensis*, *Phoenix dactylifera* and *Trachycarpus fortunei*. In addition, the present report adds a new insight into the distribution of *Morinia* in Europe, where most of the previous collections were done, reporting it for the first time from Portugal. The presence of *Morinia* species on palm trees in Portugal suggests that this genus may be widespread in Europe. Although palms are typical tropical and subtropical plants, in the present study only ornamental palms growing in a temperate climate were sampled. It is expected that new collections of angiosperms in Portugal may reveal new *Morinia* species. This would help to populate the phylogenetic delimitation of this genus within the *Sporocadaceae*.

Only six species are described in *Morinia* and only a few strains of each species have been isolated since it was first reported in 1889. In the present study only two strains of both *M. trachycarpae* and *M. phoenicicola* were isolated from palms. Although its phylogenetic position is solid, a greater number of sequences should be added to the sequences database, which will strengthen the generic boundaries between *Morinia* and phylogenetic related genera. This is particularly important considering that the intergeneric relationships in *Sporocadaceae* have been subject to multiple rearrangements in the past decades, in part due to the limited sampling of the majority of genera and, subsequently, inadequate molecular data (Jeewon *et al*., 2002; Barber *et al*., 2011; Tanaka *et al*., 2011; Senanayake *et al*., 2015; Jaklitsch *et al*., 2016; Wijayawardene *et al*. 2016; Hongsanan *et al*., 2017; Liu *et al*., 2019). In addition, some generic complexes in *Sporocadaceae*, such as *Pestalotiopsis-Truncatella-Morinia* and *Seimatosporium-Sarcostroma-Diploceras*, possess similar morphological characters of conidia and appendages, which caused difficulties in the intergeneric classification based on morphology, highlighting the importance of acquiring molecular data from a larger number of strains (Liu *et al*., 2019).

Besides the molecular data, the new morphological characters reported in this study also reveal further distinctions within *Morinia* and from other genera in *Sporocadaceae*. *Morinia trachycarpae* produces globose enclosed pycnidial structures that have not previously been reported in the genus. Liu *et al*. (2019) suggested that *Morinia*, along with *Heterotruncatella* and *Hymenopleella*, was one of the most equivocal generic concepts in *Sporocadaceae* due to its interspecific variable morphological characters. This morphological plasticity was found in *M. trachycarpae* and *M. phoenicicola*, which were particularly heterogeneous with respect to conidial morphology. While *M. trachycarpae* has subcylindrical hyaline transversely septate conidia with a wedge-shaped apical cell resembling *M. acaciae, M. phoenicicola* has fusiform versicoloured transversely septate conidia with a hemispherical-shaped apical cell and apical appendages disposition resembling *M. longiappendiculata* and *M. pestalozzioides*, although these two species have muriformly septate conidia. The present results, along with the previous ones, on these morphological characters regarding the conidial septation and apical cell shape suggest the existence of two morphological groups within *Morinia*, although they are not supported by phylogenetic data. In fact, phylogenetically ITS and LSU sequences data suggest that the *M. acaciae-M. phoenicicola* clade is closer to the *M. longiappendiculata-M. pestalozzioides* clade than to the *M. crini-M. trachycarpae* clade.

Considering the previous status of *Morinia* as mostly an endophytic fungus, its association with foliar lesions may reveal *Morinia* as a latent pathogen in angiosperms. In fact, *M. pestalozzioides* has been reported causing spots on dead stems of *Artemisia campestris* in Poland (Adamska, 2007). Since *Morinia* is a member of *Sporocadaceae*, where many antifungals have been discovered (Wang *et al*., 2016), including *Morinia* (Basilio *et al*., 2006; Vicente *et al.*, 2009), the description of two new *Morinia* species in this study may represent a major step to screen for novel metabolites in future studies.

Key to species in *Morinia*

1. Conidia muriform	2
1. Conidia with transverse septa	3
2. Basal appendages ≤ 10 µm long	*Morinia pestalozzioides*
2. Basal appendages > 10 µm long	*Morinia longiappendiculata*
3. Conidia hyaline to subhyaline	4
3. Conidia versicoloured	5
4.Conidia hyaline and < 30 µm long	*Morinia trachycarpae*
4. Conidia subhyaline and ≥ 30 µm long	*Morinia acaciae*
5. Conidia 4-septate	*Morinia crini*
5. Conidia 3-septate	*Morinia phoenicicola*

Case study III. *Arecamyces humilianae* gen. *et* sp. nov., a new *Teratosphaeriaceae* genus and species from palms in Portugal

1. Introduction

Dothideomycetes OE Erikss. & Winka (Eriksson and Winka, 1997) comprises heterogeneous and phylogenetically diverse range of fungi characterized by bitunicate asci, with fissitunicate dehiscence (Schoch *et al*. 2009; Videira *et al*., 2017). Many of these fungi have a global distribution, occurring in a wide range of habitats and lifestyles, from saprobes to phytopathogens and endophytes (Hyde *et al*., 2013; Vicente *et al*., 2017). Currently *Dothideomycetes* includes more than 25 orders, 100 families and over 1500 genera (Schoch *et al*. 2009; Hyde *et al*. 2013; Crous *et al*., 2015; Hernández-Restrepo *et al*., 2016; Krisai-Greilhuber *et al*., 2017; Videira *et al*., 2017; Liu *et al*., 2018; Crous *et al*., 2019). Among them, the order *Capnodiales* Woron. (Woronichin, 1925) includes 9 families (Videira *et al*., 2017), one of which is *Teratosphaeriaceae* Crous & U. Braun (Crous *et al*., 2007).

Teratosphaeriaceae was established by Crous *et al*. (2007) to accommodate the type genus *Teratosphaeria* Syd. & P. Syd. (Sydow and Sydow, 1912) previously included in *Mycosphaerellaceae* Lindau (Engler and Prantl, 1897). This was based on morphological characters, distinct asexual morphs and molecular data. Presently, *Teratosphaeriaceae* encompasses several genera of saprobes, opportunistic human pathogens and lichens (Quaedvlieg *et al*., 2014). Several genera of extremophiles have been linked to this family, including rock-inhabiting fungi (Ruibal *et al*., 2009; Hyde *et al*., 2013; Egidi *et al*., 2014; Trovão *et al*., 2019) and heat-treated and extreme acidic soils fungi (Seifert *et al*., 2004b; Hujslová *et al*., 2013). Furthermore, species of *Teratosphaeriaceae*, along with *Mycosphaerellaceae*, represent important leaf spotting pathogens and are often reported as *Teratosphaeriaceae* leaf diseases (TLD) and *Mycosphaerellaceae* leaf diseases (MLD) (Hunter *et al*., 2006; Crous *et al*., 2008, 2009a; Pérez *et al*., 2009, 2013; Taylor *et al*., 2012; Quaedvlieg *et al*., 2014).

The phylogeny of *Teratosphaeriaceae* has been frequently re-evaluated (Crous *et al*., 2009b,c,d; Videira *et al*., 2016), but many lineages remain unresolved and are treated as *Teratosphaeria* sp. or *Teratosphaeriaceae* (Ruibal *et al*., 2009, 2011; Egidi *et al*., 2014). Most recently, Quaedvlieg *et al*. (2014) applied the consolidated species concept (CSC) via a polyphasic approach to circumscribe most *Teratosphaeriaceae* clades and other closely associated and unclassified families, initially regarded as *Teratosphaeriaceae* "1" and "2". Subsequently, two new families have been introduced to accommodate these taxa, *Neodevriesiaceae* Quaedvl. & Crous and *Extremaceae* Quaedvl. & Crous, which were further arranged and expanded by Crous *et al*. (2015), Isola *et al*. (2016), Wang *et al*. (2017), and Delgado *et al*. (2018). Further collections may add additional morphological characters to the unnamed generic clades in *Teratosphaeriaceae*, many of which remain poorly understood and greatly undersampled.

In the present study, an interesting *Mycosphaerella*-like fungus was found associated with leaf spot symptoms on *Chamaerops humilis* in Oeiras, Portugal. The aim of this study was to characterize the fungus in terms of morphology and determine its phylogenetic position in *Teratosphaeriaceae* based on analysis of ITS and LSU sequence data.

2. Materials and Methods

All the methods used here were the same as those described in Case Study I except that only ITS and LSU loci were included in the phylogenetic analyses.

Phylogenetic analyses were done on a matrix of concatenated ITS and LSU sequences of representative genera in *Teratosphaeriaceae*. These were retrieved from GenBank according to the BLAST result for each locus and to the recent available literature on *Teratosphaeriaceae*, such as Quaedvlieg *et al*. (2014), Isola *et al*. (2016), Wang *et al*. (2017) and Delgado *et al*. (2018). Sequences obtained from GenBank are listed by their accession numbers, while newly generated sequences are listed by their isolate number and can be accessed in Supplementary Table A.12. The newly generated sequences will be deposited in GenBank. Sequences of *Capnodium coffeae* Pat. were used as the outgroup taxon.

3. Results

3.1. Phylogenetic analyses

The available ITS and LSU sequences of 82 strains of *Capnodiales*, either sequenced in this study or retrieved from GenBank, were included in the phylogenetic analysis. The concatenated ITS and LSU alignment of 81 ingroup and 1 outgroup taxa comprised 1156 characters including alignment gaps. Of the 1156 characters, 653 were constant and 139 variable characters were parsimony-uninformative. MP analysis of the remaining 364 parsimony-informative characters resulted in 140 equally parsimonious trees of 2181 steps and a relatively high level of homoplasy as indicated by a CI of 0.381, a RI of 0.693, a HI of 0.619 and a RC of 0.264. The topology of the trees differed from one another only in the position of the isolates within the terminal groupings of the *Teratosphaeria* clade. All the other clades were consistent in their phylogenetic positions. Tree topologies resulting from maximum parsimony and maximum likelihood analyses were similar and both presented well-resolved clades for each genus included in the analyses, supported by a high bootstrap value (≥ 70%). The ML tree is shown in Figure 3.18, with bootstrap support values above the branches. Although well-resolved, the phylogenetic position among the different clades within *Teratosphaeriaceae* received low bootstrap support (≤ 50). Nevertheless, *Teratosphaeriaceae* and *Neodevriesiaceae* were well-separated, insomuch that the later represents a clade with 100% bootstrap support, which confirms the phylogenetic difference that supports these two families.

The *Mycosphaerella*-like isolate obtained in this study clustered in a completely separate and previously undescribed lineage among the selected genera in *Teratosphaeriaceae* and *Neodevriesiaceae*. Nevertheless, its placement between these two families received a low bootstrap support. Considering the results from both MP and ML analyses, this fungus clusters closer to *Teratosphaeriaceae* genera. A total of 9 and 5 unique base pairs differences in the ITS and LSU loci, respectively, among the 82 isolates included in the phylogenies, confirms the novel lineage as a new genus here introduced in *Capnodiales*.

3.2. Taxonomy

Based on DNA phylogeny, the *Mycosphaerella*-like isolate collected from foliar diseases of palms was distinct from the previous *Mycosphaerellaceae* and *Teratosphaeriaceae* genera described. The present data indicate that this fungus resides in *Teratosphaeriaceae* as a new genus and new species and descriptions are provided below. Furthermore, a new TLD from *Chamaerops humilis* is reported.

Arecamyces DRS Pereira & AJL Phillips gen. nov.
MycoBank: MBXXX

Etymology: named after the host family from which it was collected, *Arecaceae*.

Type species: ***Arecamyces humilianae*** DRS Pereira & AJL Phillips sp. nov.

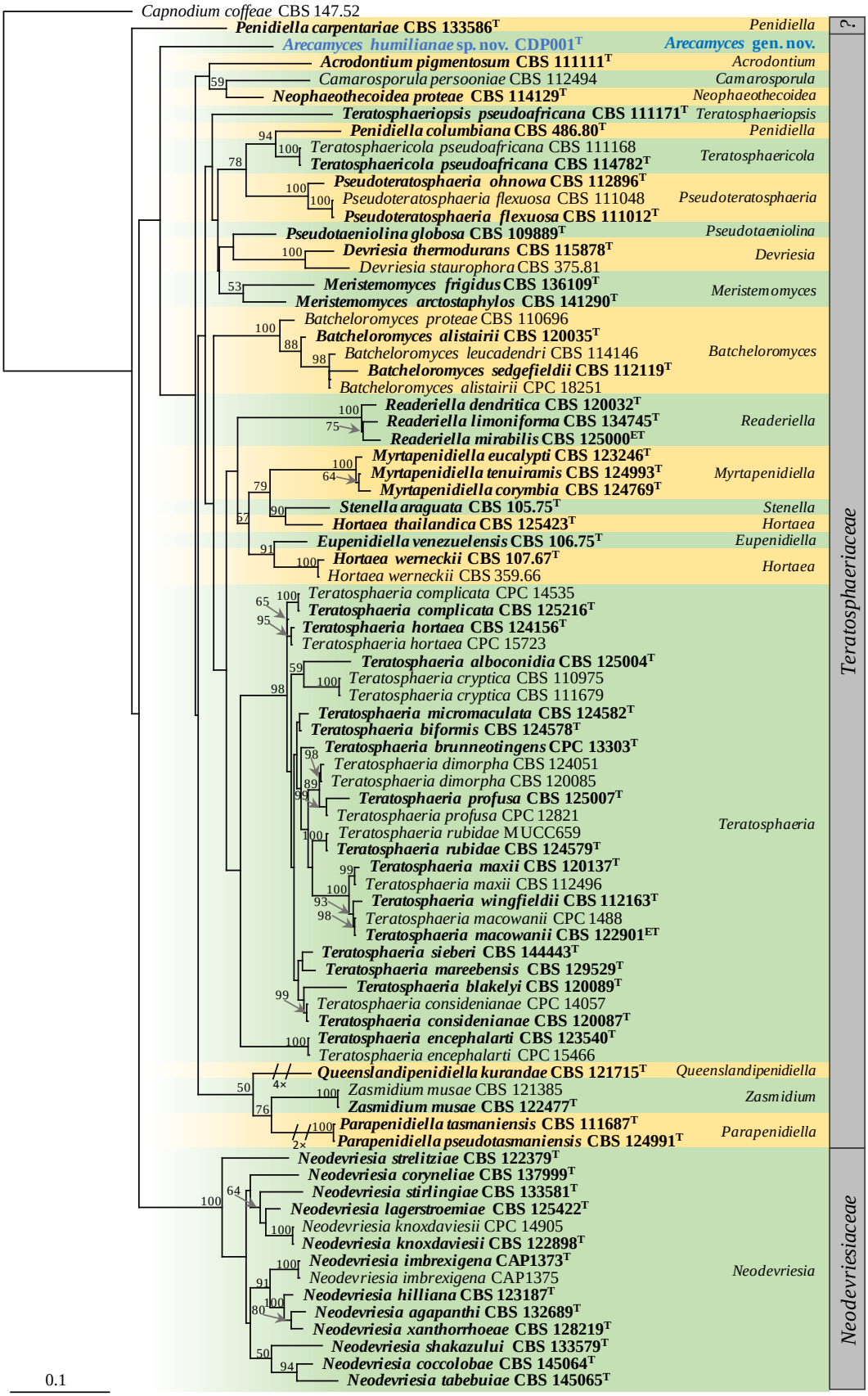

Figure 3.18 Phylogenetic position of *Arecamyces humilianae*. ML tree generate by RAxML with GTR+G+I nucleotide substitution model using the combined two loci ITS-LSU. ML bootstrap support values (> 50%) are shown above the branches. The isolate from this study is listed in blue. The scale bar represents the expected number of nucleotide changes per site. *Capnodium coffeae* (CBS 147.52) was included as outgroup. Ex-type (T)/ex-epitype (ET)/ cultures are marked in bold.

Ascomata pseudothecial, amphigenous, subepidermal, immersed to erumpent, scattered or clustered, globose to subglobose, dark-brown, ostiolate. *Ostiole* circular, aperiphysate. *Peridium* thin-walled, composed of cells forming a *textura angularis*, outer layer composed of thick-walled, dark-brown to brown cells, inner layers composed of thin-walled, hyaline cells. *Pseudoparaphyses* absent. *Asci* bitunicate, fissitunicate, pyriform to obovoid, slightly curved, broader at the apex, well-developed ocular chamber, smooth-walled, hyaline, 8-spored, biseriate. *Ascospores* broadly ellipsoidal to cylindrical, with rounded ends, smooth- and thin-walled, medianly 1-septate, not constricted at the septum.

Arecamyces humilianae DRS Pereira & AJL Phillips sp. nov. (Figure 3.19)
MycoBank: MBXXXX

Etymology: named after the host genus from which it was collected, *Chamaerops humilis*.

Leaf spots sunken, circular to broadly ellipsoidal, 3–7 × 2–3 mm (mean ± SD = 4.67 ± 1.06 × 2.52 ± 0.51 mm), identical on both leaf surfaces, brown-grey to yellowish center, later becoming greyish and fragile, with dark-brown border (ca. 1 mm wide), surrounded by a conspicuous brown to red-brown halo, occasionally coalesce, randomly distributed. Mature spots contain several immersed ascomata. *Ascomata* pseudothecial, amphigenous, subepidermal, immersed to erumpent, scattered or clustered in groups of 2 or 3, globose to subglobose, dark-brown, up to 90 µm diam (n = 6), ostiolate. *Ostiole* circular, up to 21 µm diam (n = 6), aperiphysate. *Peridium* thin-walled, composed of cells forming a *textura angularis*, outer layer composed of thick-walled, dark-brown to brown cells, inner layers composed of thin-walled, hyaline cells. *Pseudoparaphyses* absent, but pseudoparenchymatous, cellular hamathecium remnant present. *Asci* bitunicate, outer wall up to 2 µm thick, fissitunicate, pyriform to obovoid, slightly curved, broader at the apex, well-developed ocular chamber, smooth-walled, hyaline, 8-spored, biseriate, 21.4–57.9 × 8.2–13.2 µm (mean ± SD = 32.25 ± 14.99 × 10.76 ± 2.21 µm, n = 5). *Ascospores* broadly ellipsoidal to cylindrical, with rounded ends, occasionally slightly curved, smooth- and thin-walled, medianly 1-septate, not constricted at the septum, 9.5–17.9 × 2.8–4.0 µm (mean ± SD = 14.41 ± 2.49 × 3.35 ± 0.31 µm, n = 22); mean ± SD ascospore length/width ratio = 4.31 ± 0.71 (n = 22).

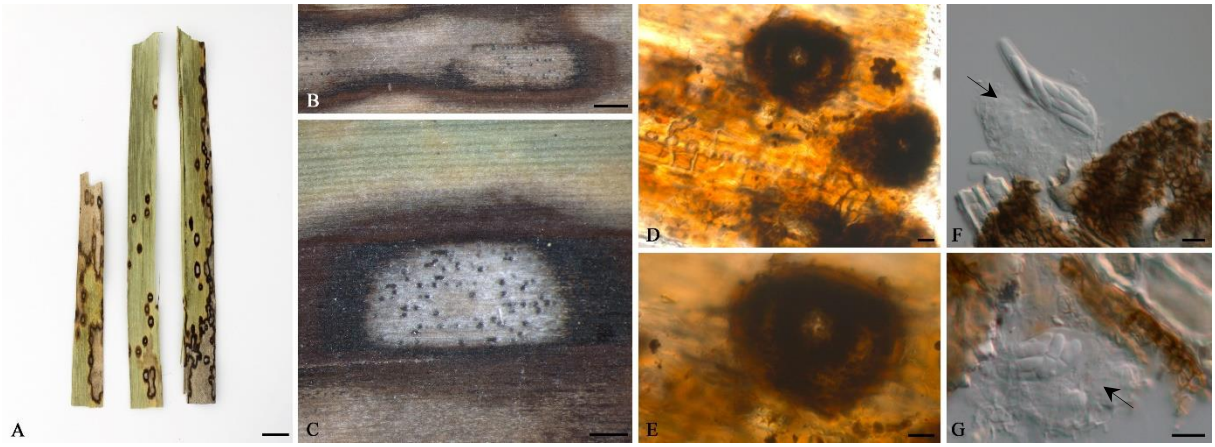

Figure 3.19 ***Arecamyces humilianae*** **(ex-type CDP 001)**. **A**. Leaf spots on segments of *Chamaerops humilis*. **B – E**. Appearance of ascomata on host surface. **F**, **G**. Asci, cellular hamathecium remnants (black arrows) and ascospores. Scale bars: A = 10 mm, B, C = 1 mm, D – E = 15 µm, F, G = 10 µm.

Material examined: Portugal, Oeiras, União das Freguesias de Algés, Linda-a-Velha e Cruz Quebrada – Dafundo, National Sports Centre of Jamor, on leaf spots of *Chamaerops humilis* (*Arecaceae*), 20 September 2018, Alan JL Philips (specimen/holotype HDP 003, ex-type culture CDP 001, ITS sequence SDP 001/01, LSU sequence SDP 001/02).

Distribution: Oeiras, Portugal.

Hosts: *Chamaerops humilis*.

Notes: *Arecamyces humilianae* was found associated with leaf spots of *Chamaerops humilis*, but pathogenicity has not been tested. Nevertheless, there is evidence that this species represents an obligate biotroph causing a new disease on *C. humilis*. The fungus barely grew on culture, attaining 1 mm diam after 1 month of incubation. Furthermore, this growth corresponded to a black, amorphous mass of globose sterile mycelium, which could hardly be referred to as a colony. Although phylogenetically closer to *Teratosphaeriaceae* genera (Figure 3.18), and thus here included in this family, *A. humilianae* highly resembles *Mycosphaerella* Johanson (Johanson, 1884) sexual morphs (Figure 3.19), with small, inconspicuous, roughly globose ascomata which become erumpent or immersed in the host tissue, bitunicate asci, absence of pseudoparaphyses and hyaline, medianly 1-septate ascospores (Fröhlich and Hyde, 1998a).

4. Discussion

In the present study, a new species in *Teratosphaeriaceae*, *Arecamyces humilianae*, is described and a new genus is established to accommodate this fungus. Phylogenetic analyses based on ITS and LSU sequences revealed that *Arecamyces* represents a separate lineage close to several *Teratosphaeriaceae* genera, as well as to *Neodevriesiaceae*. The evidence gained from unique nucleotide differences among the several genera included in the phylogeny supports this novelty at genus-level.

Morphologically *Arecamyces humilianae* resembles *Mycosphaerellaceae* and *Teratosphaeriaceae* as characterized by small, inconspicuous ascomata immersed in the host tissue, which produce pyriform asci with 8, hyaline, ellipsoidal and medianly 1-septate ascospores. The presence of pseudoparenchymatal remnants in ascomata of *Arecamyces* and the absence of paraphyses place it within *Teratosphaeriaceae* since Crous *et al.* (2007) used these characters to separate *Teratosphaeriaceae* from *Mycosphaerellaceae*. This was confirmed by the phylogenetic analyses, which showed that *A. humilianae* is closer to *Teratosphaeriaceae* and *Neodevriesiaceae* genera than to *Mycosphaerellaceae* genera. Nevertheless, the low bootstrap support for the branches leading to *A. humilianae* suggest that future studies may reveal a different phylogenetic position for this taxon. Genera in *Teratosphaeriaceae* and *Mycosphaerellaceae* are often defined based not only on DNA sequence data, but also on morphology of their asexual morphs. However, *A. humilianae* barely grew in culture and no signs of asexual sporulation could be detected. This is common in *Mycosphaerella*, and *Teratosphaeria* species, which are cultivated with difficulty (Crous *et al*., 2007).

The phylogenetic position of *A. humilianae* is still undetermined and no accurate nearest neighbours can be point out in this analysis. Sequences from only two loci, ITS and LSU, were used in the present study and thus the uncertainty of phylogenetic position may be related to the lack of molecular data. Most recent studies that aimed to clarify *Teratosphaeriaceae*, as well as *Mycosphaerellaceae*, clades used a combination of additional loci to ITS and LSU, which include for example the actin-like protein (ACT), the translation elongation factor 1-alpha (TEF1) and the β-tubulin (TUB2) genes (e.g. Hunter *et al*., 2006; Pérez *et al*., 2013; Crous *et al*., 2018, 2019). Thus, it is possible that the use of other DNA barcodes may help to clarify the phylogenetic position of *Arecamyces* among other genera in *Teratosphaeriaceae*. In addition, a high level of homoplasy was detected with MP analysis. This is not surprising, considering that most genera within *Teratosphaeria* are polyphyletic and within *Capnodiales* are paraphyletic (Crous *et al*., 2007). In fact, convergence is observed in several genera especially concerning the morphology of asexual morphs (Crous *et al*., 2007, 2009b; Ruibal *et al*., 2008; Quaedvlieg *et al*., 2014).

Although it is not assumed as a new family in the present study due to lack of data, especially concerning a greater number of isolates, the phylogenetic analyses in this study predicts that *Arecamyces* will be. Besides the DNA phylogenetic data, *A. humilianae* lacks several morphological characters that are diagnostic for *Teratosphaeria*, the type genus of *Teratosphaeriaceae*. These include ascospores that turn brown and verruculose while still in the ascus as well as the presence of mucoid sheaths around the ascospores (Quaedvlieg *et al*., 2014). Thus, *Arecamyces* represents another new genus within *Capnodiales*, where several phylogenetic lineages remain poorly resolved due to limited sampling (Quaedvlieg *et al*., 2014).

However, its position within *Teratosphaeriaceae* cannot be confirmed and it is possible that future studies with greater taxon sampling may eventually split *Arecamyces* from *Teratosphaeriaceae*.

Arecamyces was collected from diseased foliage of *Chamaerops humilis* and represents a new insight into the *Teratosphaeriaceae* leaf diseases (TLD) and *Mycosphaerellaceae* leaf diseases (MLD). Although the pathogenicity of *A. humilianae* has not been tested, its extremely slow growth rate and almost lack of growing on agar may suggest that this fungus presents highly specific requirements and can be reported as an obligate biotroph. This extremely slow growth rate is often reported in important leaf spotting fungal within *Capnodiales* (Crous *et al*., 2008) and it seems clearly that *Arecamyces humilianae* represents a new record of a phytopathogenic fungi. The report of a new leaf spotting fungi in *Capnodiales* represents a significant advance in the TLD and MLD knowledge, since these fungi are important phytopathogens in various plant hosts, such as *Eucalyptus* (Hunter *et al*., 2006; Crous *et al*., 2009a; Pérez *et al*., 2009, 2013; Taylor *et al*., 2012; Quaedvlieg *et al*., 2014). Furthermore, several species within *Capnodiales* families, especially *Teratosphaeriaceae*, are of quarantine importance to many countries in Europe (Crous *et al*., 2009a; Quaedvlieg *et al*., 2012). Future studies should aim to better understand the ecology and physiology of *Arecamyces* in order to assess its pathogenicity traits as a phytopathogen. Its geographical distribution is, for now, confined to a single plant in Portugal. Therefore, further sampling is essential to understand its geographical and ecological range.

Global Discussion and Final Remarks

Palm trees continue to present a diverse assemblage of fungi, many of which are new to science (Fröhlich and Hyde, 2000; Hyde *et al*., 2000; Taylor and Hyde, 2003; Pinnoi *et al*., 2006; Pinruan *et al*., 2007; Rungjindamai *et al*., 2008; Konta *et al*., 2016c; Zang *et al*., 2018, 2019). In that respect, it is not surprising that *Diaporthe chamaeropsicola*, *Morinia trachycarpae*, *Morinia phoenicicola* and *Arecamyces humilianae* have been found as new species on the palms in Portugal. Although this study was based on a relatively small sampling, it clearly confirms the already reported high diversity of palm mycobiota.

To search for new fungal species is of utmost importance. Although fungi are an essential and useful group of organisms with an enormous biotechnological potential for industrial exploitation, they are relatively understudied (Hyde *et al*., 2019b). Considering the actual estimates of global fungal species – 2.2 to 3.8 million – and that only about 120 000 species are presently known and named, less than 10% of the worldwide mycota is described (Hawksworth and Lücking, 2017). Moreover, pondering that over the past 40 years new fungal species were reported at an average rate of 1 300 to 1 800 per year, it is expected that it will take more than 2 000 years before all the missing fungi are named (Hawksworth and Lücking, 2017). Therefore, the world has a huge wealth of undiscovered and unexploited microfungi that could hold great potential for mankind. A representative example is discussed to illustrate this potential.

Morinia, along with *Pestalotiopsis* and *Bartalinia*, is a member of *Sporocadaceae*, a family of particular interest because of their production of secondary metabolites (Basilio *et al*., 2006; Collado *et al*., 2006; Liu *et al*., 2019). For example, *Pestalotiopsis fici* was shown to possess a great number of gene clusters involved with the synthesis of bioactive compounds (Wang *et al*., 2016). Considering that genera in *Sporocadaceae* share the same evolutionary history, other species in this family are expected to have a high potential to produce secondary metabolites similar to that found in *Pestalotiopsis*. Thus, many novel metabolites might be hidden and await discovery in members of *Sporocadaceae* (Vicente *et al*., 2009; Liu *et al*., 2019) and this include *Morinia* species. In fact, Basilio *et al*. (2006) reported a new antifungal compound produced by *M. pestalozzioides*, moriniafungin, a sordarin with a broad antifungal spectrum not found in any other member of *Sporocadaceae* or any other fungal family (Vicente *et al*., 2009). In addition, one of the sequences retrieved from GenBank belonged to an isolate of *M. phoenicicola* that was previously isolated from living plant material as an endophyte (Weber *et al*., 2007). This isolate was accommodated in *Morinia phoenicicola* in the present study and it is known to be a producer of arundifungin. Thus, it is probable that the *Morinia phoenicicola* isolates from the present work are also capable of producing bioactive compounds of this kind.

This example emphasizes how important it is to look for new fungal species, especially concerning the bioactive compounds that these unknown microorganisms can hold. Considering that only 3% of the isolates

in the present study have been fully characterized, more novel species are expected to be found if the other isolates are studied in-depth.

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APPENDICES

Appendix A. Taxonomic diversity of palmiculous fungi

Appendix B. General workflow

Appendix C. Genera-abundance distribution

Appendix D. Foliar lesions types of different host species

Appendix E. Composition of host species fungal assemblages

Appendix F. Collector's effort curve

Appendix G. Composition of parishes fungal assemblage

Appendix H. Foliar lesions types and host species examined on different parishes

Appendix I. Distribution of fungal occurrences on foliar lesions of palms

Appendix J. Co-occurrence analyses

Appendix K. Reproductibility system of the genomic diversity

Appendix L. Nucleotide differences among taxa

Appendix M. Details of the sequences

Appendix N. Coelomycetes presumptive identification details

Appendix O. Overview of morphological diversity

Appendix A. Taxonomic diversity of palmiculous fungi

Supplementary Table A.1 Genera with common representatives found on palms.

Genera	References
Anthostomella	Hyde, 1994f, 1996c; Hyde *et al*., 1998b
Appendicospora	Hyde, 1995g; Yanna *et al.*, 1997
Appendispora	Hyde, 1994a, 1999a
Arecomyces	Hyde, 1996g
Arecophila	Hyde, 1996f
Astrosphaeriella	Hyde, 1992a, 1993d, 1994e, 2000; Hyde and Fröhlich, 1997
Capsulospora	Hyde, 1996d
Fasciatispora	Hyde, 1991b, 1995c; Lu and Hyde, 1999
Guignardia	Hyde 1995e
Helicascus	Hyde, 1991a
Leptosphaeria	Hyde, 1992a
Lignicola	Hyde, 1992a
Linocarpon	Hyde, 1988, 1992a,b, 1997b; Hyde and Alias, 1999; Konta *et al.*, 2017
Mycosphaerella	Hyde and Fröhlich, 1995b; Fröhlich and Hyde, 1998a
Myelosperma	Hyde, 1993f
Neodeightonia	Phillips *et al*., 2008; Liu *et al.*, 2010; Konta *et al.*, 2016a
Neolinocarpon	Hyde, 1992a; Hyde *et al*., 1998a; Hyde and Alias, 1999; Konta *et al*., 2017
Nipicola	Hyde, 1992d, 1994b
Oxydothis	Hyde KD and Nakagiri A, 1989; Hyde, 1993d, 1994c; Hidayat *et al.*, 2006; Konta *et al*., 2016b
Palmicola	Hyde, 1993b; Goh and Hyde, 1996a
Pemphidium	Hyde, 1993a, 1996e
Phomatospora	Hyde, 1993d
Seynesia	Hyde 1995f

Supplementary Table A.2 Genera described as new to science and found on palms in the last three decades.

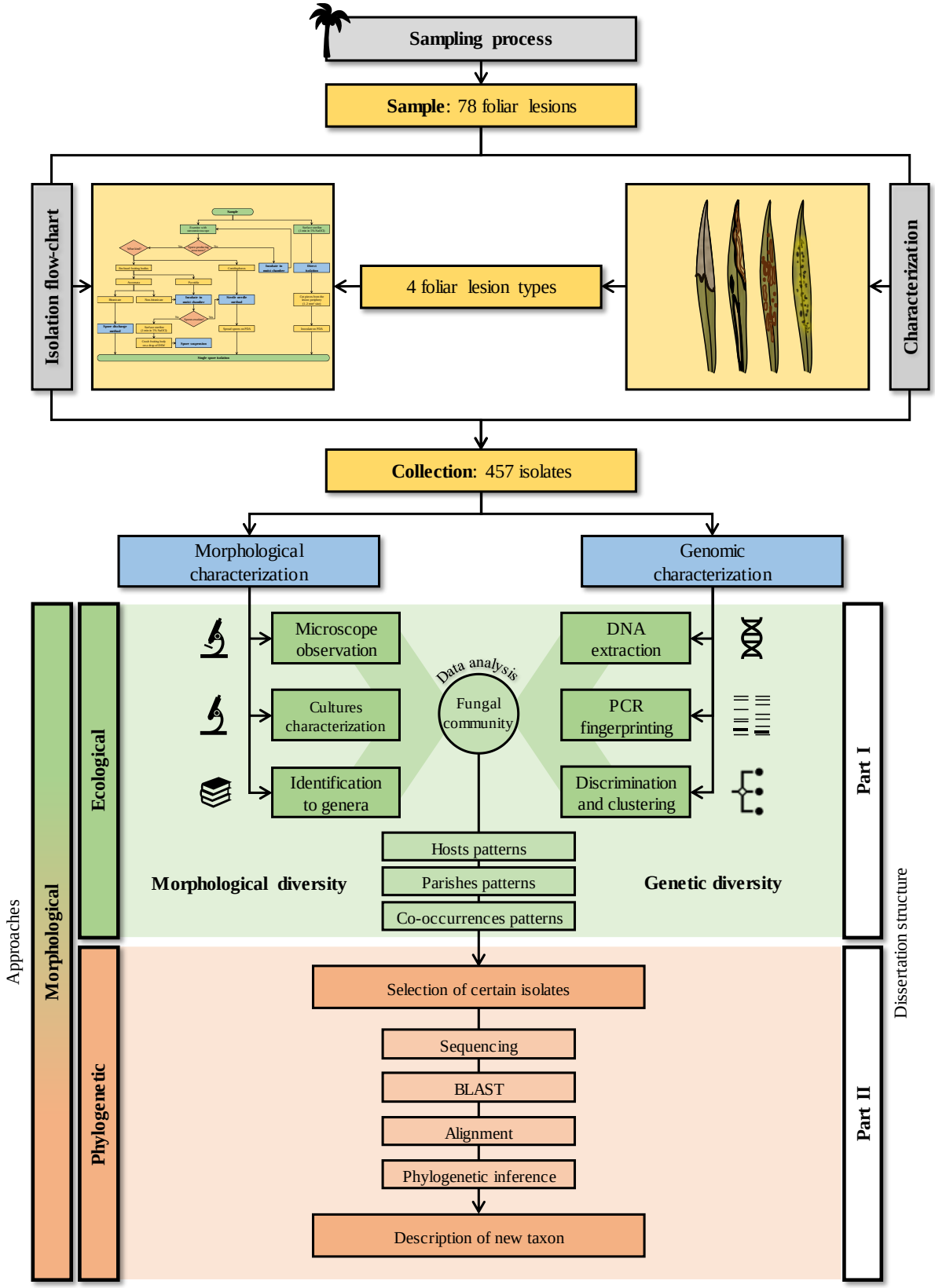
Genera	Host*	Region*	Reference
Acuminatispora	*Phoenix paludosa*	Thailand	Zhang *et al.*, 2018
Apioclypea	*Livistona* sp.	Papua New Guinea	Hyde, 1994f
Appendicospora	*Corypha utan*	Philippines	Hyde, 1995d
Appendispora	*Oncosperma horridum*	Brunei	Hyde, 1994a
Arecacicola	*Calamus* sp.	Indonesia (Java)	Taylor *et al.*, 2001
Arecomyces	*Arenga undulatifolia*	Brunei	Hyde, 1996g
Arecophila	*Gulubia costata*	Papua New Guinea	Hyde, 1996f
Asymmetricospora	*Calamus caryotoides*	Australia (North Queensland)	Fröhlich and Hyde, 1998b
Baipadisphaeria	*Licuala longecalycata*	Thailand	Pinruan *et al.*, 2010
Bharatheeya	*Calamus thwaitesii*	India	D'Souza and Bhat, 2002
Brachysporiopsis	*Livistona chinensis*	Hong Kong	Yanna *et al.*, 2004
Brunneiapiospora	*Daemonorops* sp.	Brunei	Hyde *et al.*, 1998b
Cannonia	*Butia yatay*	Argentina	Taylor and Hyde, 1999b
Capsulospora	*Daemonorops* sp.	Brunei	Hyde, 1996d
Carinispora	*Nypa fruticans*	Brunei	Hyde, 1992a
Castanedospora	*Sabal palmetto*	USA (Florida)	Delgado *et al.*, 2018
Caudatispora	*Phytelephas* sp.	Ecuador	Fröhlich and Hyde, 1995a
Cocoicola	*Cocos nucifera*	Papua New Guinea	Hyde, 1995b
Curvatispora	*Livistona spinosa*	Singapore	Sarma and Hyde, 2001
Durispora	*Elaeis guineensis*	Malaysia	Hyde, 1994d
Endosporoideus	*Phoenix hanceana*	Hong Kong	Ho *et al.*, 2005
Ernakulamia	*Cocos nucifera*	India	Subramanian, 1996
Fasciatispora	*Nypa fruticans*	Brunei	Hyde, 1991b
Fissuroma	*Arenga westerhoutii*	Thailand	Liu *et al*., 2011b
Flammispora	*Licuala longecalycata*	Thailand	Pinruan *et al.*, 2004d
Fondispora	*Chamaerops humilis*	Italy	Hyde, 1993d
Frondicola	*Nypa fruticans*	Brunei	Hyde, 1992a
Guestia	*Mauritia flexuosa*	Ecuador	Smith and Hyde, 2001
Kalamarospora	*Sabal palmetto*	USA (Florida)	Delgado, 2010
Lockerbia	Unknown palm specie	Australia (North Queensland)	Hyde, 1993h
Longicorpus	*Nypa fruticans*	Thailand	Zang *et al*., 2019
Mackenziella (as *Mackenziea*)	*Oraniopsis appendiculate*	Australia (North Queensland)	Yanna and Hyde, 2002
Maculatifrondis	Unknown palm specie	Ecuador	Hyde *et al.*, 1996b
Maculatipalma	*Linospadix microcaryus*	Australia (North Queensland)	Fröhlich and Hyde, 1995d
Manokwaria	Unknown palm specie	Indonesia (West Papua)	Hyde, 1993g
Neoastrosphaeriella	*Metroxylon sagu*	Thailand	Liu *et al*., 2011b
Neolinocarpon	*Nypa fruticans*	Brunei	Hyde, 1992a
Nipicola	*Nypa fruticans*	Brunei	Hyde, 1992d
Nusia	*Scheelea insignis*	Malaysia (Singapore)	Subramanian, 1993

Supplementary Table A.2 Continued.

Genera	Host*	Region*	Reference
Ornatispora	*Calamus conirostris*	Australia (North Queensland)	Hyde *et al.*, 1999d
Palmicola	*Archontophoenix alexandrae*	Australia (North Queensland)	Hyde, 1993b
Palmomyces	*Oraniopsis appendiculate*	Australia (North Queensland)	Kevin *et al*., 1998b
Phruensis	*Licuala longecalycata*	Thailand	Pinruan *et al.*, 2004c
Polybulbophiale	*Licuala* sp.	Brunei	Goh and Hyde, 1998c
Pulmosphaeria	*Archontophoenix alexandrae*	Australia (North Queensland)	Taylor *et al.*, 1996
Rachidicola	*Calamus* sp.	Hong Kong	Hyde and Fröhlich, 1995a
Sabalicola	*Sabal serrulate*	USA (Florida)	Hyde, 1995a
Stratiphoromyces	*Licuala* sp.	Brunei	Goh and Hyde, 1998b
Striatiguttula	*Nypa fruticans*	Thailand	Zang *et al.*, 2019
Thailandiomyces	*Licuala longecalycata*	Thailand	Pinruan *et al.*, 2008
Tirisporella	*Nypa fruticans*	Malaysia	Jones *et al.*, 1996
Tretendophragmia	*Korthalsia* sp.	Malaysia (Singapore)	Subramanian, 1992
Tretocephala	*Oncosperma horridum*	Malaysia (Singapore)	Subramanian, 1995
Unisetosphaeria	*Eleiodoxa conferta*	Thailand	Pinnoi *et al*., 2003a
Veramycella	*Sabal palmetto*	USA (Florida)	Delgado, 2009
Waihonghopes	*Oraniopsis appendiculate*	Australia (North Queensland)	Yanna and Hyde, 2002

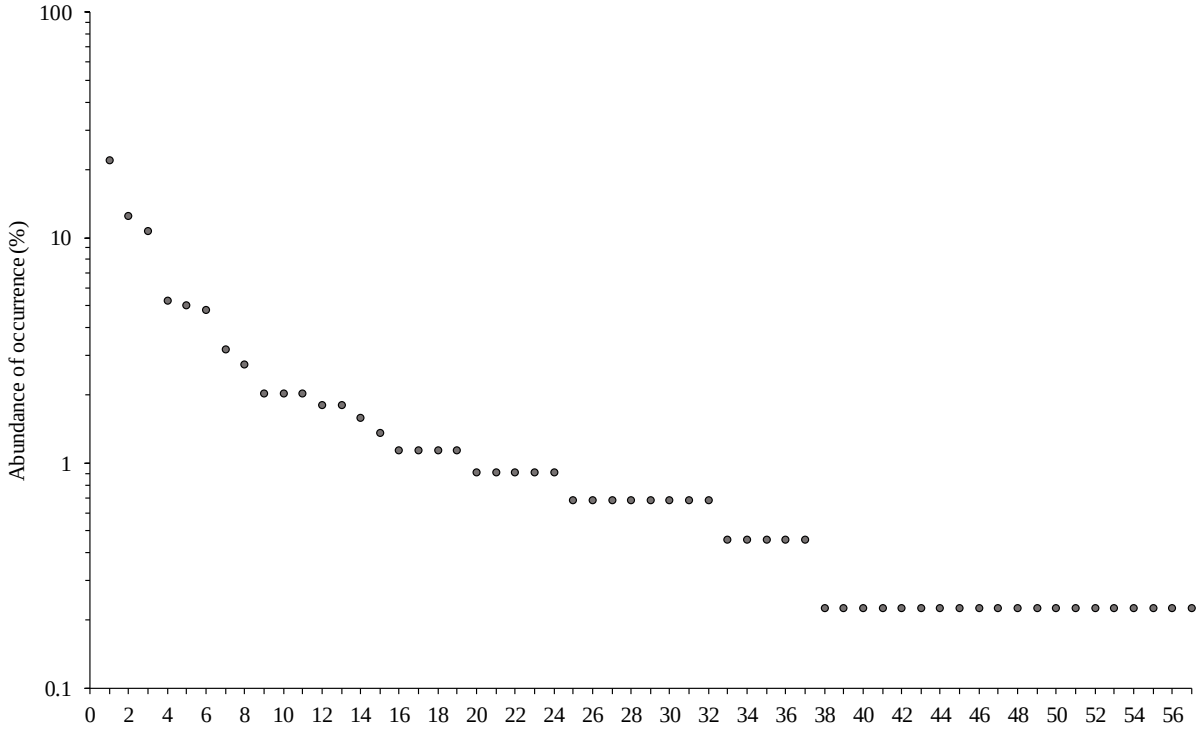
*Related to the type species and to the holotype, when possible.

Appendix B. General workflow



Supplementary Figure A.1 Schematic overview of the general workflow.

Appendix C. Genera-abundance distribution



Supplementary Figure A.2 Abundance distributions for fungal genera associated with palm foliar lesions. The y-axis (log scale) represents the abundance of occurrence of each genus, which is the relative importance of each genus in the fungal assemblage as a percent. The sum of all relative importance values equals 100%. Each genus is ranked from the most abundant to least abundant along the x-axis.

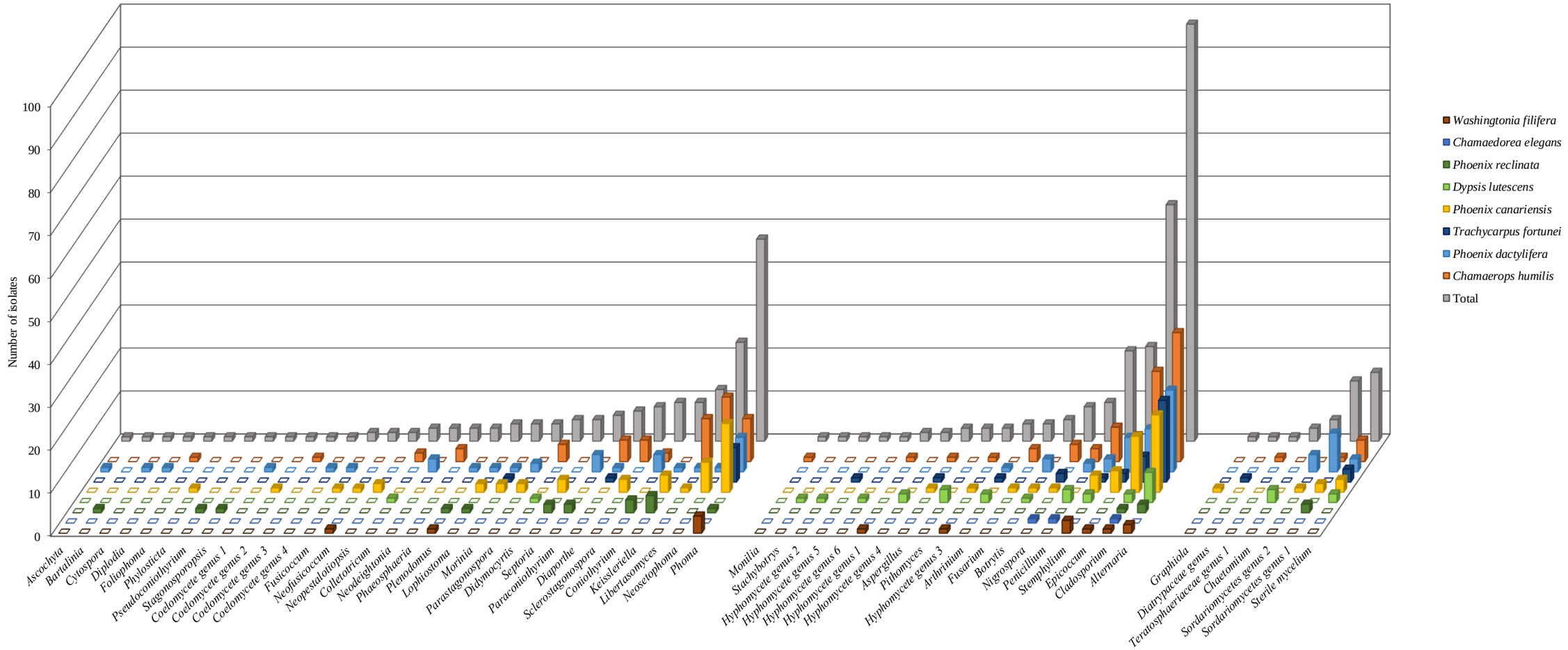
Appendix D. Foliar lesions types of different host species

Supplementary Table A.3 Absolute frequencies of foliar lesions types per host species.

Host	**TDB**[*]	**LLS**[#]	**SLS**[†]	**PP**[‡]
Chamaerops humilis	6	3	2	10
Dypsis lutescens	1	3	2	1
Phoenix canariensis	6	4	8	8
Phoenix dactylifera	1	4	2	5
Trachycarpus fortunei	3	2	1	0

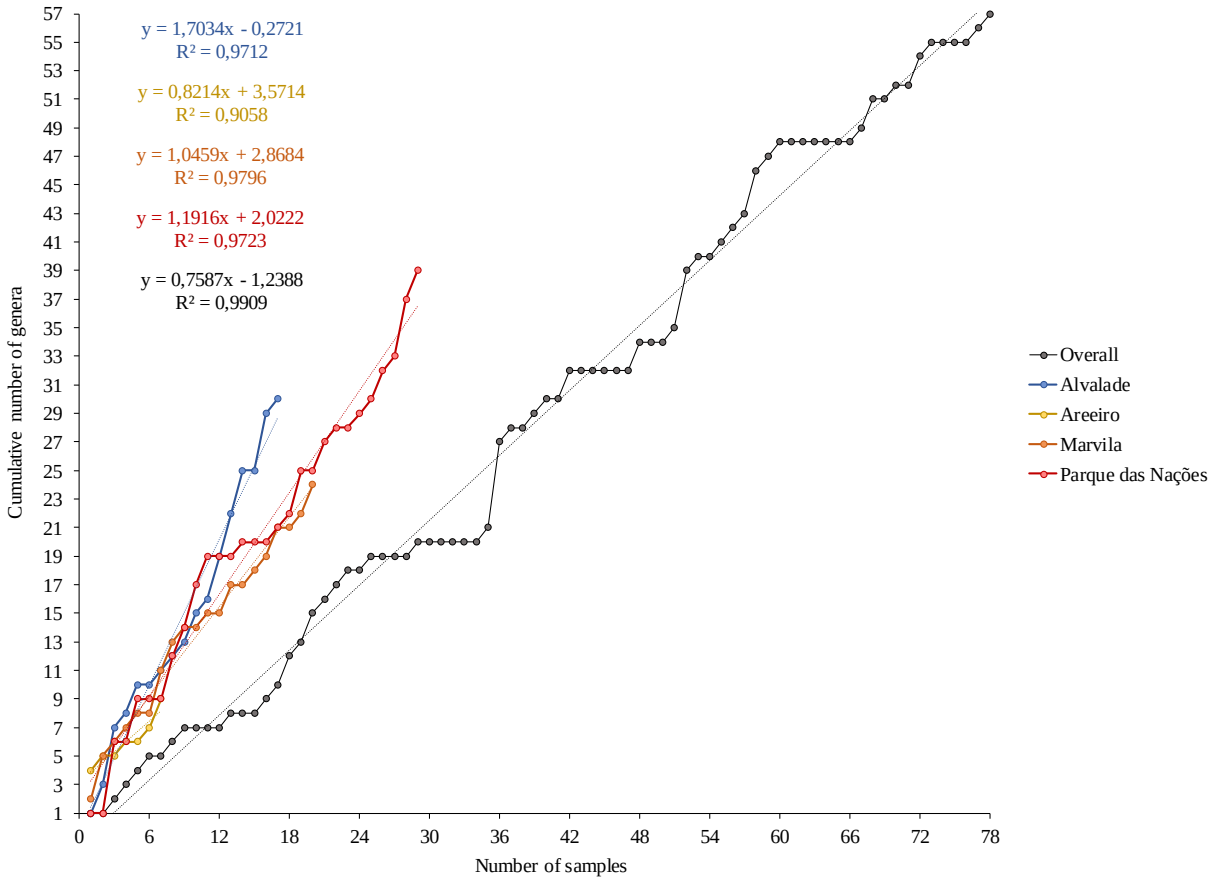
[*]TDB, tip die-back.
[#]LLS, large leaf spot.
[†]SLS, small leaf spot.
[‡]PP, pinpoints and punctuations.

Appendix E. Composition of host species fungal assemblages



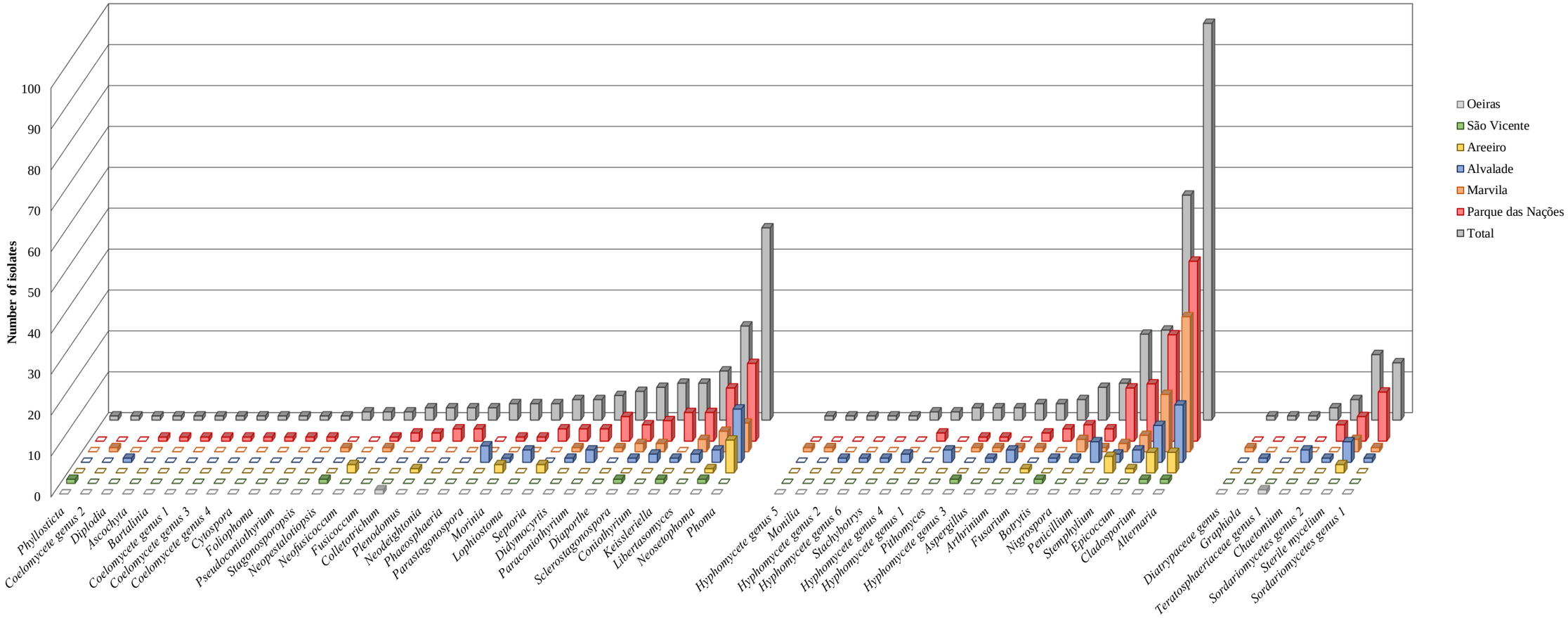
Supplementary Figure A.3 Absolute frequencies of the number of isolates per genus and per host species.

Appendix F. Collector's effort curve



Supplementary Figure A.4 Collector's effort curve for the cumulative number of genera recovered from each parish and from the overall sample set. Incremental increase in the number of genera isolated plotted against the number of samples examined in each parish and for the overall sample set. Data from different parishes and for the overall sample set were plotted in random order. Solid lines represent the calibration curves for each genera-accumulation curve plotted and are shown along with the corresponding equation. Colours are according to parishes.

Appendix G. Composition of parishes fungal assemblage



Supplementary Figure A.5 Absolute frequencies of the number of isolates per genus and per parish.

Appendix H. Foliar lesions types and host species examined on different parishes

Supplementary Table A.4 Absolute frequencies of foliar lesions types per parish.

Host	TDB[*]	LLS[#]	SLS[†]	PP[‡]
Alvalade	5	5	4	3
Areeiro	1	3	2	3
Marvila	8	3	2	7
Parque das Nações	5	7	6	9

[*]TDB, tip die-back.
[#]LLS, large leaf spot.
[†]SLS, small leaf spot.
[‡]PP, pinpoints and punctuations.

Supplementary Table A.5 Absolute frequencies of trees from each host species per parish.

Host	Alvalade	Areeiro	Marvila	Parque das Nações
Chamaedorea elegans	1	0	0	0
Chamaerops humilis	0	0	5	7
Dypsis lutescens	5	0	0	0
Phoenix canariensis	3	4	4	2
Phoenix dactylifera	3	0	0	3
Phoenix reclinata	0	0	0	2
Trachycarpus fortunei	0	1	2	2
Washingtonia filifera	0	1	1	1

Appendix I. Distribution of fungal occurrences on foliar lesions of palms

Supplementary Table A.6 Distribution of fungal occurrences on foliar lesions of palms. Number of samples supporting n fungal isolates per sample and the corresponding absolute frequency of isolates.

Fugal records (n) per sample	Break-up of number of samples examined	Total fungal occurrences*
n = 1	10	10
n = 2	12	24
n = 3	8	24
n = 4	10	40
n = 5	10	50
n = 6	8	48
n = 7	2	14
n = 8	3	24
n = 9	2	18
n = 10	1	10
n = 12	3	36
n = 14	1	14
n = 15	3	45
n = 16	2	32
n = 17	1	17
n = 23	1	23
n = 28	1	28

*Total fungal occurrences, number of samples × n.

Appendix J. Co-occurrence analyses

Supplementary Table A.7 Patterns of fungal genera co-occurrence on palm foliar lesions.

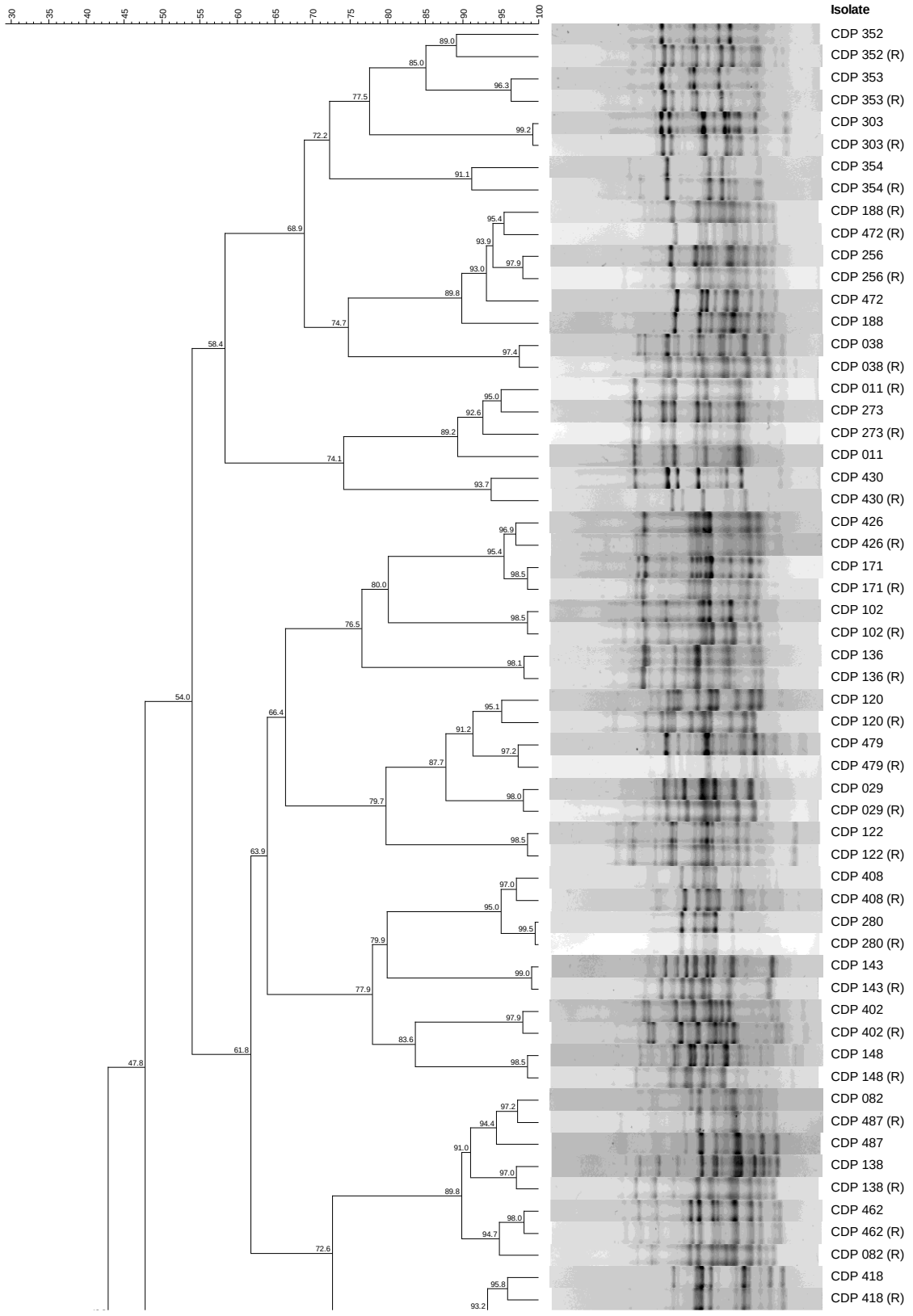
Genera	Number of samples in which		Total occurrences	FO*; FCO#	Number of other genera that co-occur with each genus
	a genus co-occurs	a genus occurs singly			
Alternaria	37	1	38	48.72; 78.95	45
Arthrinium	4	0	4	5.13; 22.81	13
Ascochyta	1	0	1	1.28; 12.28	7
Aspergillus	3	0	3	3.85; 10.53	6
Bartalinia	1	0	1	1.28; 12.28	7
Botrytis	5	0	5	6.41; 10.04	8
Chaetomium	2	0	2	2.56; 7.02	4
Cladosporium	32	3	35	44.87; 68.42	39
Collectotrichum	1	1	2	2.56; 12.28	7
Coniothyrium	5	0	5	6.41; 29.82	17
Lophiostoma	4	0	4	5.13; 24.56	14
Cytospora	1	0	1	1.28; 8.77	5
Diaporthe	6	0	6	7.69; 26.32	15
Didymocyrtis	3	1	4	5.13; 21.05	12
Diplodia	1	0	1	1.28; 10.53	6
Epicoccum	13	0	13	16.67; 42.11	24
Foliophoma	1	0	1	1.28; 7.02	4
Fusarium	3	0	3	3.85; 12.28	7
Fusicoccum	2	0	2	2.56; 7.02	4
Graphiola	1	0	1	1.28; 5.26	3
Keissleriella	5	0	5	6.41; 29.82	17
Libertasomyces	7	0	7	8.97; 24.56	14
Monilia	1	0	1	1.28; 7.02	4
Morinia	3	1	4	5.13; 12.28	7
Neodeightonia	3	0	3	3.85; 12.28	7
Neofusicoccum	2	0	2	2.56; 8.77	5
Neopestalotiopsis	2	0	2	2.56; 12.28	7
Neosetophoma	11	1	12	15.38; 31.58	18
Nigrospora	5	0	5	6.41; 19.30	11
Paraconiothyrium	3	0	3	3.85; 15.79	9
Parastagonospora	2	0	2	2.56; 8.77	5
Penicillium	8	0	8	10.26; 28.07	16
Phaeosphaeria	2	0	2	2.56; 17.54	10
Phoma	31	1	32	41.03; 59.65	34
Phyllosticta	1	0	1	1.28; 1.75	1
Pithomyces	1	0	1	1.28; 10.53	6
Plenodomus	3	0	3	3.85; 19.30	11
Pseudoconiothyrium	1	0	1	1.28; 12.28	7

Supplementary Table A.7 Continued.

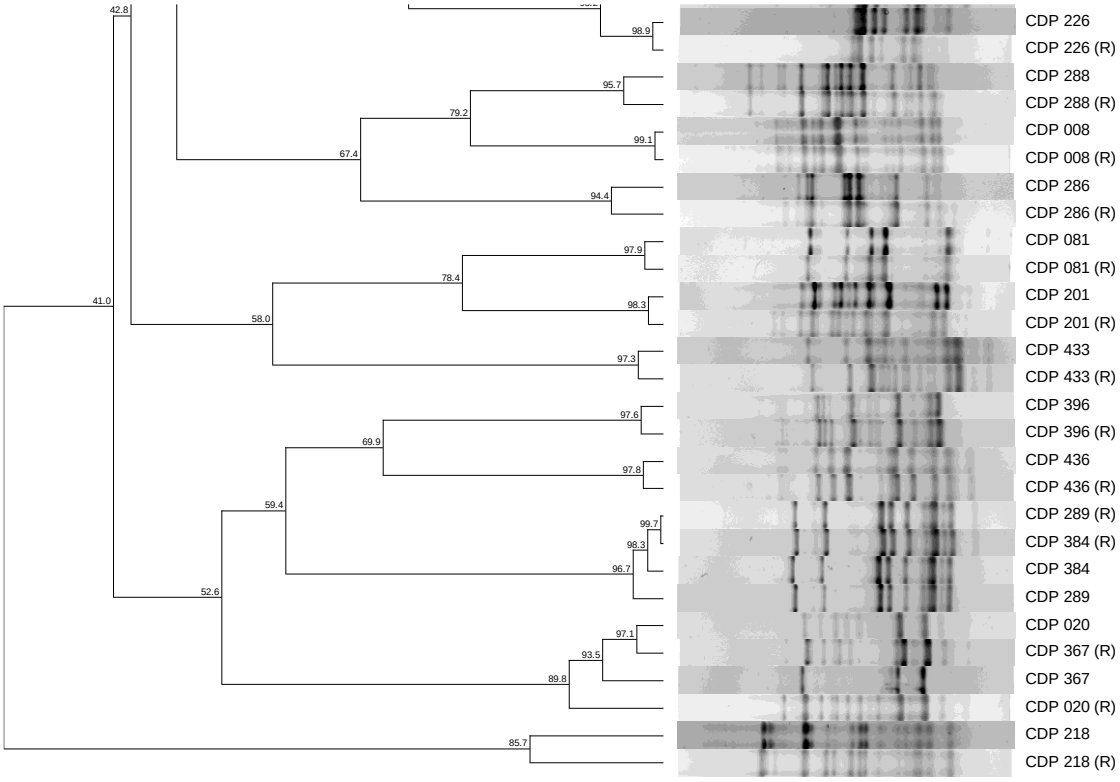
Genera	Number of samples in which		Total occurrences	FO*; FCO#	Number of other genera that co-occur with each genus
	a genus co-occurs	a genus occurs singly			
Sclerostagonospora	7	0	7	8.97; 29.82	17
Septoria	5	0	5	6.41; 29.82	17
Stachybotrys	1	0	1	1.28; 5.26	3
Stagonosporopsis	1	0	1	1.28; 12.28	7
Stemphylium	14	0	14	17.95; 47.37	27
Coelomycete genus 1	1	0	1	1.28; 1.75	1
Coelomycete genus 2	1	0	1	1.28; 1.75	1
Coelomycete genus 3	1	0	1	1.28; 7.02	4
Coelomycete genus 4	1	0	1	1.28; 14.04	8
Diatrypaceae genus	1	0	1	1.28; 7.02	4
Teratosphaeriaceae genus 1	0	1	1	1.28; 0.00	0
Hyphomycete genus 1	2	0	2	2.56; 12.28	7
Hyphomycete genus 2	1	0	1	1.28; 3.51	2
Hyphomycete genus 3	3	0	3	3.85; 10.53	6
Hyphomycete genus 4	2	0	2	2.56; 15.79	9
Hyphomycete genus 5	1	0	1	1.28; 8.77	5
Hyphomycete genus 6	1	0	1	1.28; 10.53	6
Sordariomycetes genus 1	8	0	8	10.26; 38.60	22
Sordariomycetes genus 2	4	0	4	5.13; 15.79	9

*FO, frequency of occurrence (%).
#FCO, frequency of co-occurrence (%).

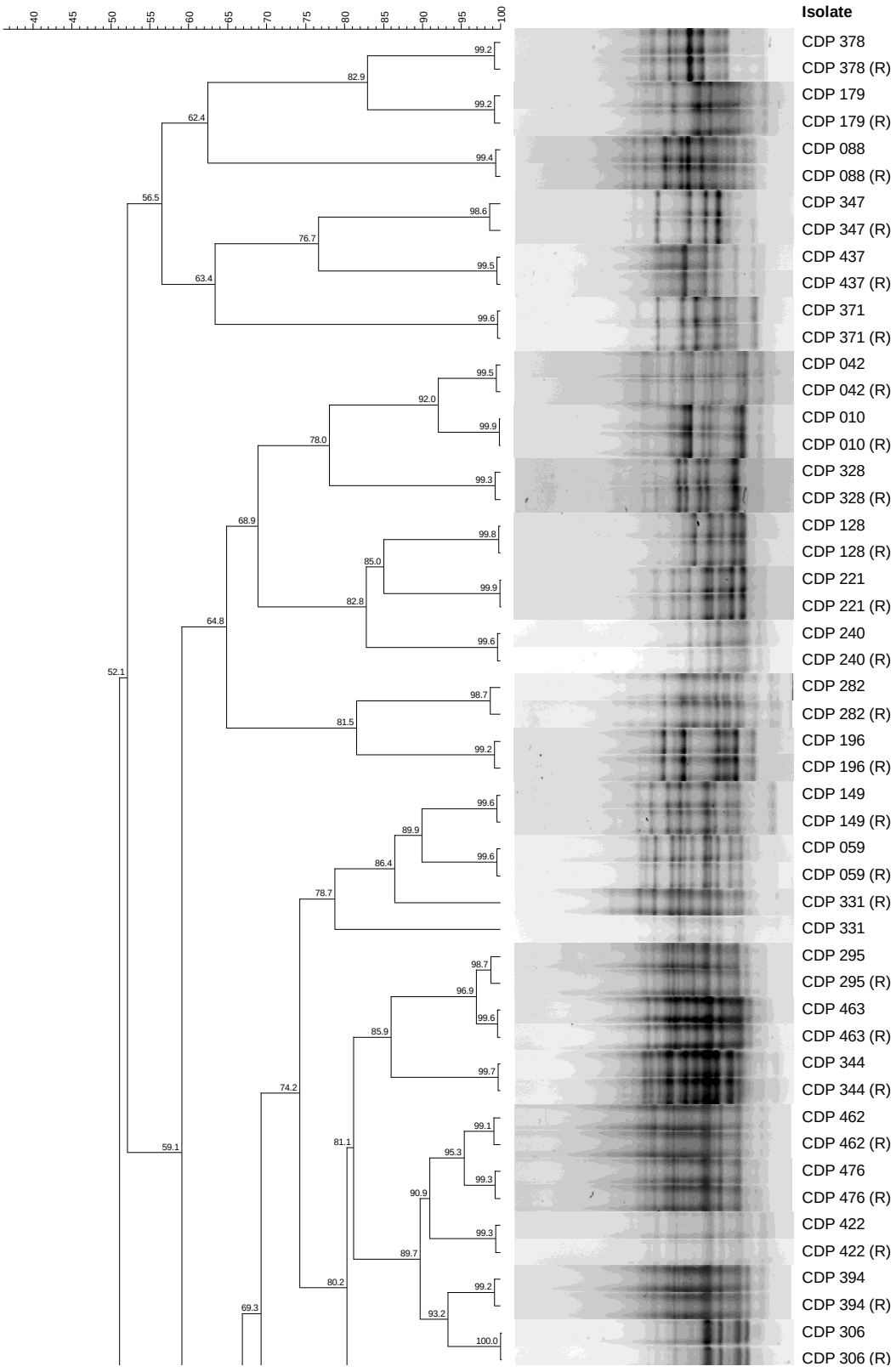
Appendix K. Reproductibility system of the genomic diversity



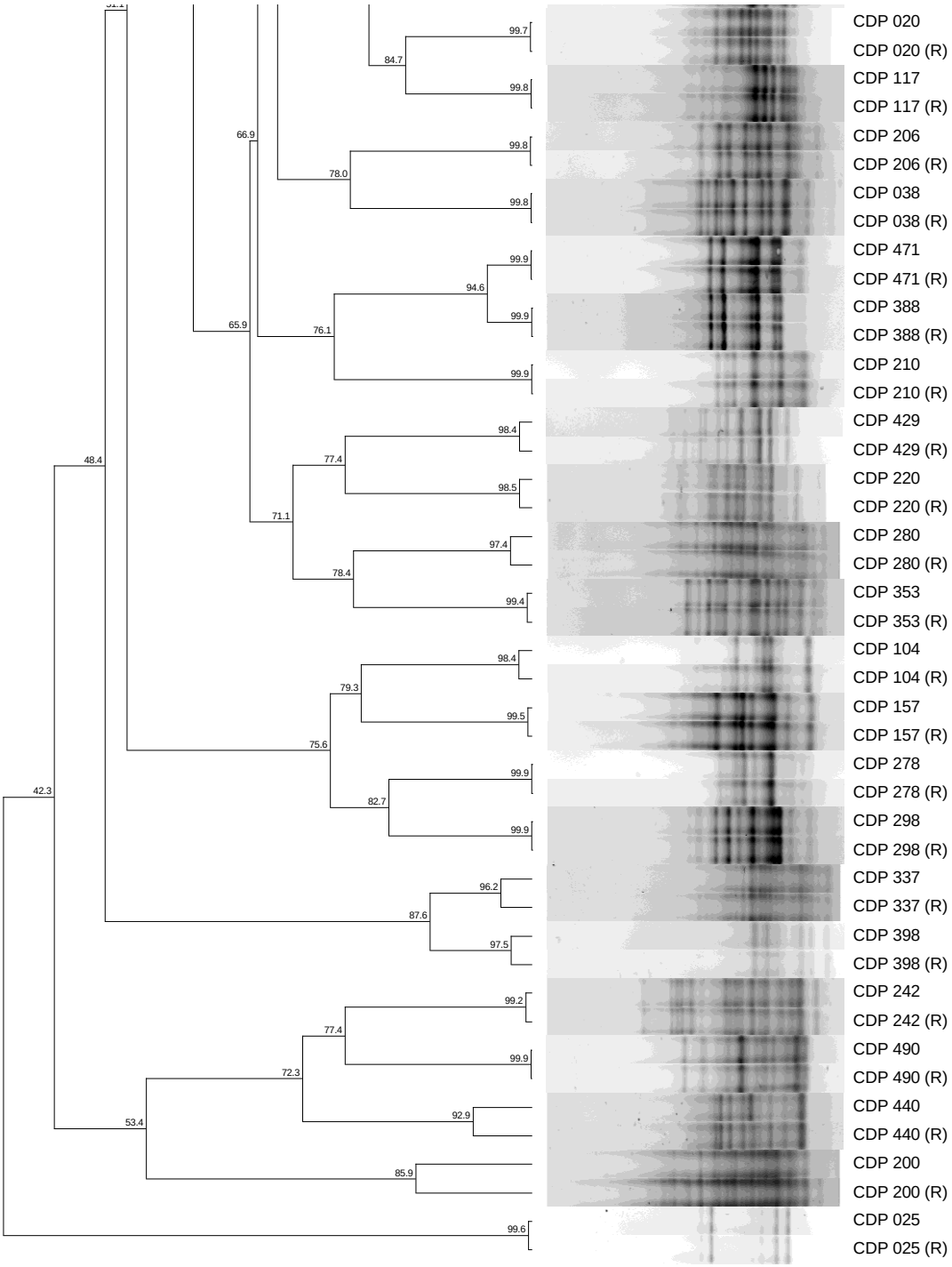
Supplementary Figure A.6 Reproducibility gels for csM13 profiles. Dendrogram based on csM13 profiles performed in BioNumerics using Pearson's correlation coefficient and UPGMA. The numbers of the isolates next to which (R) is written represent the replicates, while the others represent the original profiles obtained.



Supplementary Figure A.6 Continued.



Supplementary Figure A.7 Reproducibility gels for $(GTG)_5$ profiles. Dendrogram based on $(GTG)_5$ profiles performed in BioNumerics using Pearson's correlation coefficient and UPGMA. The numbers of the isolates next to which (R) is written represent the replicates, while the others represent the original profiles obtained.



Supplementary Figure A.7 Continued.

Appendix L. Nucleotide differences among taxa

Supplementary Table A.8 Nucleotide differences between *Diaporthe chamaeropsicola*, *D. ceratozamiae*, *D. phyllanthicola* and *D. loropetali*.

Locus	Position	*D. chamaeropsicola*	*D. ceratozamiae*	*D. phyllanthicola*	*D. loropetali*
ITS (502 bp*)	19	C	ND#	T	T
	23	–	ND	ND	G
	58	A	–	ND	ND
	60	C	T	ND	ND
	86	T	ND	C	C
	87	C	T	ND	ND
	89	T	ND	ND	C
	93	C	ND	T	T
	98	C	T	ND	ND
	101	–	C	C	C
	102	–	C	C	C
	104	C	–	–	–
	105	C	–	–	–
	158	C	T	ND	ND
	177	–	ND	ND	A
	363	T	C	ND	ND
	390	A	ND	ND	T
	393	A	G	C	C
	442	C	T	ND	T
	461	C	T	T	ND

*bp, base pairs.

#ND, no differences.

Supplementary Table A.9 Nucleotide differences between *Morinia trachycarpae* and *M. crini*, and between *M. phoenicicola* and *M. acaciae*.

Locus	Position	*M. trachycarpae*	*M. crini*	*M. phoenicicola*	*M. acaciae*
ITS (530 bp[*])	9	G	ND[#]	G	–
	43	C	T	C	ND
	74	C	G	G	ND
	80	–	T	–	ND
	110	C	ND	C	T
	112	G	ND	G	A
	122	A	ND	A	T
	135	C	ND	C	A
	143	C	T	C	ND
	146	G	ND	G	A
	147	T	C	C	ND
	155	T	C	T	ND
	173	–	ND	–	T
	410	T	C	T	ND
	412	A	G	A	ND
	417	G	ND	G	T
	426	C	ND	C	T
	473	T	ND	T	C
	498	A	ND	A	G
	514	T	A	T	ND
	525	–	A	A	ND
	530	C	ND	C	T
LSU (571 bp)	461	G	A		
	468	–	A		
	473	–	T	NA[†]	
	490	A	T		
	491	C	T		
	563	G	C		

[*]bp, base pairs.
[#]ND, no differences.
[†]NA, not available.

Appendix M. Details of the sequences

Supplementary Table A.10 Isolates used in the phylogenetic analyses of *Diaporthe*.

Taxon	Culture[1]	Status[2]	GenBank accession number[3]			
			ITS*	TUB2*	TEF1*	CAL*
Diaporthe arecae	CBS 161.64	IT	KC343032	KC344000	KC343758	KC343274
D. arecae	CBS 535.75		KC343033	KC344001	KC343759	KC343275
D. arecae	PBMR345		MK111088	MK122810	MK117275	–
D. arengae	CBS 114979	T	KC343034	KC344002	KC343760	KC343276
D. aspalathi	CBS 117168		KC343035	KC344003	KC343761	KC343277
D. aspalathi	CBS 117169	T	KC343036	KC344004	KC343762	KC343278
D. aspalathi	CBS 117500		KC343037	KC344005	KC343763	KC343279
D. baccae	CBS 136972	T	KJ160565	MF418509	KJ160597	–
D. baccae	CPC 30315		MG281014	MG281187	MG281535	MG281709
D. beilharziae	BRIP 54792	T	JX862529	KF170921	JX862535	–
D. caulivora	CBS 127268	NT	KC343045	KC344013	KC343771	KC343287
D. caulivora	CBS 178.55		KC343046	KC344014	KC343772	KC343288
D. ceratozamiae	CBS 131306	T	JQ044420	–	–	–
D. chamaeropsicola	CDP 460/01	T	**SDP 460/01**	**SDP 460/02**	**SDP 460/03**	**SDP 460/04**
D. chimonanthi	SCHM 3614	T	AY622993	–	–	–
D. cytosporella	AR5149		KC843309	KC843222	KC843118	KC843287
D. cytosporella	FAU461	ET	KC843307	KC843221	KC843116	KC843141
D. eucalyptorum	CBS 132525	T	JX069862	–	–	–
D. foeniculina	CBS 111553	T	KC343101	KC344069	KC343827	KC343343
D. foeniculina	CBS 123209		KC343105	KC344073	KC343831	KC343347
D. foeniculina	CBS 187.27		DQ286287	JX275463	DQ286261	KC843122
D. foeniculina	CDP 022		**SDP 022/01**	**SDP 022/02**	**SDP 022/03**	–
D. foeniculina	CDP 209		**SDP 209/01**	**SDP 209/02**	**SDP 209/03**	–
D. foeniculina	CDP 315		**SDP 315/01**	**SDP 315/02**	**SDP 315/03**	–
D. helianthi	CBS 592.81	T	KC343115	KC344083	KC343841	JX197454
D. helianthi	CBS 344.94		KC343114	KC344082	KC343840	KC343356
D. hongkongensis	CBS 115448	T	KC343119	KC344087	KC343845	KC343361
D. hongkongensis	ZJUD74		KJ490609	KJ490430	KJ490488	–
D. infecunda	CBS 133812	T	KC343126	KC344094	KC343852	KC343368
D. infecunda	CMT60		KP182394	KP182402	KP182384	KP182376
D. infecunda	LGMF 908		KC343127	KC343853	KC344095	KC343369
D. leucospermi	CBS 111980	T	JN712460	KY435673	KY435632	KY435663
D. liquidambaris	SCHM 3621	T	AY601919	–	–	–
D. liquidambaris	TW15		MH930416	–	–	–
D. longicolla	FAU599	T	KJ590728	KJ610883	KJ590767	KJ612124
D. longicolla	FAU 644		KJ590730	KJ610885	KJ590769	KJ612126
D. loropetali	SCHM 3615	T	AY601917	–	–	–
D. musigena	CBS 129519	T	KC343143	KC344111	KC343869	KC343385
D. musigena	HKFZL006		MK050110	MK079660	MK054238	–
D. novem	CBS 127269		KC343155	KC343881	KC344123	KC343397

Supplementary Table A.10 Continued.

Taxon	Culture[1]	Status[2]	GenBank accession number[3]			
			ITS*	TUB2*	TEF1*	CAL*
D. novem	CBS 127270	T	KC343156	KC343882	KC344124	KC343398
D. novem	CBS 127271		KC343157	KC344125	KC343883	KC343399
D. pascoei	BRIP 54847	IT	JX862532	KF170924	JX862538	–
D. pascoei	PBMR348		MK111093	MK122809	MK117276	–
D. phaseolorum	AR4203	ET	KJ590738	KJ590739	KJ610893	–
D. phaseolorum	CBS 127465		KC343177	KC344145	KC343903	KC343419
D. phyllanthicola	SCHM 3680	T	AY620819	–	–	–
D. pseudomangiferae	CBS 101339	T	KC343181	KC344149	KC343907	KC343423
D. pseudomangiferae	CBS 388.89		KC343182	KC344150	KC343908	KC343424
D. pseudophoenicicola	CBS 176.77		KC343183	KC344151	KC343909	KC343425
D. pseudophoenicicola	CBS 462.69	T	KC343184	KC344152	KC343910	KC343426
D. pseudophoenicicola	CDP 047		**SDP 047/01**	**SDP 047/02**	**SDP 047/03**	**SDP 047/04**
D. pseudophoenicicola	CDP 358		**SDP 358/01**	**SDP 358/02**	**SDP 358/03**	**SDP 358/04**
D. pyracanthae	CAA483	T	KY435635	KY435666	KY435625	KY435656
D. pyracanthae	CAA487		KY435636	KY435667	KY435626	KY435657
D. pyracanthae	CDP 052		**SDP 052/01**	**SDP 052/02**	**SDP 052/03**	**SDP 052/04**
D. rhusicola	CBS 129528	T	JF951146	KC843205	KC843100	KC843124
D. sackstonii	BRIP 54669b	T	KJ197287	KJ197267	KJ197249	–
D. serafiniae	BRIP 55665a	T	KJ197274	KJ197254	KJ197236	–
D. serafiniae	BRIP 54136		KJ197273	KJ197253	KJ197235	–
D. sojae	CBS 116019		KC343175	KC344143	KC343901	KC343417
D. sojae	DP0601		KJ590706	KJ610862	KJ590749	KJ612103
D. sojae	FAU635	ET	KJ590719	KJ610875	KJ590762	KJ612116
D. toxica	CBS 534.93	T	KC343220	KC344188	KC343946	KC343462

[1]BRIP: Queensland Plant Pathology Herbarium, Brisbane, Australia, CBS = Culture Collection of the Westerdijk Fungal Biodiversity Institute, Utrecht, The Netherlands, CDP = Culture Collection of Diogo Pereira, housed at the Lab Bugworkers | M&B-BioISI | Tec Labs – Innovation Centre, Faculty of Sciences, University of Lisbon, Lisbon, Portugal, CPC = Culture Collection of Pedro Crous, housed at the Westerdijk Institute, FAU = Isolates in culture collection of Systematic.

[2]Status of the strains, ET = ex-epitype, H = holotype, IT = ex-isotype, NT = ex-neotype, T = ex-type.

[3]Newly generated sequences are in bold, – = no sequence available.

*ITS = internal transcribed spacers and intervening 5.8S nrDNA, TUB2 = partial β-tubulin gene, TEF1 = partial elongation factor 1-alpha gene, CAL = partial calmodulin gene.

Supplementary Table A.11 Isolates used in the phylogenetic analyses of *Morinia*.

Taxon	Strain number[1]	Status[2]	GenBank accession number[3]	
			ITS*	LSU*
Bartalinia bella	CBS 125525		GU291796	MH554214
B. bella	CBS 464.61	T	MH554051	MH554264
B. pini	CBS 143891	T	MH554125	MH554330
B. pini	CBS 144141		MH554170	MH554364
B. robillardoides	CBS 122615		MH553989	MH554207
B. robillardoides	CBS 122705	ET	LT853104	KJ710438
B. robillardoides	CDP 377		**SDP 377/01**	**SDP 377/02**
B. robillardoides	CPC 25361		MH554133	MH554335
B. rosicola	MFLUCC 17-0645	T	MG828872	MG828988
Broomella vitalbae	MFLUCC 13-0798	ET	NR_153610	KP757749
Clypeosphaeria mamillana	CBS 140735	ET	KT949897	MH554225
Heterotruncatella spartii	CPC 17945		MH554100	MH554310
Hetero. spartii	MFLUCC 15-0537	T	KR092794	KR092783
Hyalotiella spartii	MFLUCC 13-0397	T	KP757756	KP757752
Hymenopleella austroafricana	CBS 143886	T	MH554115	MH554320
Hymeno. hippophaëicola	CBS 113687		MH553969	MH554188
Hymeno. hippophaëicola	CBS 140410	ET	KT949901	MH554224
Lepteutypa sambuci	CBS 131707	T	NR_154124	MH554219
Morinia acaciae	CBS 100230		MH553950	MH554174
M. acaciae	CBS 137994	T	MH554002	MH554221
M. acaciae	E-000535696		JN545792	–
M. crini	CBS 143888	T	MH554118	MH554323
M. longiappendiculata	CBS 117603	T	AY929324	MH554202
M. pestalozzioides	ATCC No. PTA-3862	ET	AY929325	–
M. phoenicicola	CDP 191		**SDP 191/01**	–
M. phoenicicola	CDP 192/01	T	**SDP 192/01**	–
M. phoenicicola	E00175		DQ872671	–
M. trachycarpae	CDP 097/01	T	**SDP 097/01**	**SDP 097/02**
M. trachycarpae	CDP 232		**SDP 232/01**	**SDP 232/02**
Parabartalinia lateralis	CBS 399.71	T	MH554043	MH554256
Truncatella angustata	CBS 144025	NT	MH554112	MH554318
T. angustata	CBS 338.32		MH554033	MH554250
T. angustata	CBS 393.80		MH554041	MH554254
T. angustata	CBS 449.51		MH554050	MH554262

[1]ATCC = American Type Culture Collection, Virginia, USA, CBS = Culture Collection of the Westerdijk Fungal Biodiversity Institute, Utrecht, The Netherlands, CDP = Culture Collection of Diogo Pereira, housed at the Lab Bugworkers | M&B-BioISI | Tec Labs – Innovation Centre, Faculty of Sciences, University of Lisbon, Lisbon, Portugal, CPC = Culture Collection of Pedro Crous, housed at the Westerdijk Institute, MFLU(CC) = Mae Fah Luang University Culture Collection.

[2]Status of the strains, T = ex-type, ET = ex-epitype, NT = ex-neotype.

[3]Newly generated sequences are in bold, – = no sequence available.

*ITS = internal transcribed spacers and intervening 5.8S nrDNA, LSU = partial large-subunit rRNA gene.

Supplementary Table A.12 Isolates used in the phylogenetic analyses of *Arecamyces humilianae*.

Taxon	Strain number[1]	Status[2]	GenBank accession number[3]	
			ITS*	LSU*
Acrodontium pigmentosum	CBS 111111	T	KX287275	KX286963
Arecamyces humilianae	CDP 001	T	**SDP 001/01**	**SDP 002/02**
Batcheloromyces alistairii	CBS 120035	T	DQ885901	KF937220
B. alistairii	CPC 18251		JX556227	JX556237
B. leucadendri	CBS 114146		–	EU707892
B. proteae	CBS 110696		JF746163	KF901833
B. sedgefieldii	CBS 112119	T	EU707893	KF937222
Camarosporula persooniae	CBS 112494		JF770448	JF770460
Capnodium coffeae	CBS 147.52		MH856967	MH868489
Devriesia staurophora	CBS 375.81		KF442532	KF442572
D. thermodurans	CBS 115878	T	MH862991	MH874549
Eupenidiella venezuelensis	CBS 106.75	T	KF901802	KF902163
Hortaea thailandica	CBS 125423	T	MH863702	MH875167
H. werneckii	CBS 107.67	T	AJ238468	EU019270
H. werneckii	CBS 359.66		MH858825	MH870461
Meristemomyces arctostaphylos	CBS 141290	T	KX228264	KX228315
M. frigidus	CBS 136109	T	KF309971	GU250401
Myrtapenidiella corymbia	CBS 124769	T	KF901517	KF901838
M. eucalypti	CBS 123246	T	KF901772	KF902130
M. tenuiramis	CBS 124993	T	KF937262	GQ852626
Neodevriesia agapanthi	CBS 132689	T	KJ564346	JX069859
N. coccolobae	CBS 145064	T	MK047432	MK047483
N. coryneliae	CBS 137999	T	KJ869154	KJ869211
N. hilliana	CBS 123187	T	MH863277	MH874801
N. imbrexigena	CAP1373	T	JX915746	JX915750
N. imbrexigena	CAP1375		JX915748	JX915752
N. knoxdaviesii	CBS 122898	T	EU707865	EU707865
N. knoxdaviesii	CPC 14905		EU707866	KJ564328
N. lagerstroemiae	CBS 125422	T	GU214634	KF902149
N. shakazului	CBS 133579	T	KC005776	KC005797
N. stirlingiae	CBS 133581	T	KC005778	KC005799
N. strelitziae	CBS 122379	T	EU436763	GU301810
N. tabebuiae	CBS 145065	T	MK047433	MK047484
N. xanthorrhoeae	CBS 128219	T	HQ599605	HQ599606
Neophaeothecoidea proteae	CBS 114129	T	MH862955	KF937228
Parapenidiella pseudotasmaniensis	CBS 124991	T	MH863440	MH874943
Para. tasmaniensis	CBS 111687	T	KF901521	KF901843
Penidiella carpentariae	CBS 133586	T	KC005784	KC005806
Pen. columbiana	CBS 486.80	T	KF901630	KF901965
Pseudotaeniolina globosa	CBS 109889	T	KF309976	KF310010
Pseudoteratosphaeria flexuosa	CBS 111012	T	KF901755	KF902110
Pseudo. flexuosa	CBS 111048		KF901643	KF901978
Pseudo. ohnowa	CBS 112896	T	KF901620	KF901946

Supplementary Table A.12 Continued.

Taxon	Strain number[1]	Status[2]	GenBank accession number[3]	
			ITS*	ITS*
Queenslandipenidiella kurandae	CBS 121715	T	KF901538	KF901860
Readeriella dendritica	CBS 120032	T	KF901543	KF901865
R. limoniforma	CBS 134745	T	KF901547	KF901869
R. mirabilis	CBS 125000	ET	KF901549	KF901871
Stenella araguata	CBS 105.75	T	MH860897	MH872633
Zasmidium musae	CBS 122477	T	EU514291	–
Z. musae	CBS 121385		EU514293	–
Teratosphaeria alboconidia	CBS 125004	T	KF901558	KF901881
Ter. biformis	CBS 124578	T	KF901564	KF901887
Ter. blakelyi	CBS 120089	T	KF901565	KF901888
Ter. brunneotingens	CPC 13303	T	EF394853	EU019286
Ter. complicata	CBS 125216	T	MH863461	MH874961
Ter. complicata	CPC 14535		KF901781	KF902139
Ter. considenianae	CBS 120087	T	DQ923527	KF937238
Ter. considenianae	CPC 14057		KF901568	KF901892
Ter. cryptica	CBS 110975		KF901573	KF901897
Ter. cryptica	CBS 111679		KF901691	KF902037
Ter. dimorpha	CBS 120085		DQ923529	KF937240
Ter. dimorpha	CBS:124051		KF901575	KF901899
Ter. encephalarti	CBS 123540	T	FJ372395	FJ372412
Ter. encephalarti	CPC 15466		FJ372401	FJ372418
Ter. hortaea	CBS 124156	T	MH863358	MH874881
Ter. hortaea	CPC 15723		FJ790279	FJ790300
Ter. macowanii	CBS 122901	ET	MH863257	MH874781
Ter. macowanii	CPC 1488		AY260096	FJ493199
Ter. mareebensis	CBS 129529	T	JF951149	JF951169
Ter. maxii	CBS 120137	T	DQ885899	KF937243
Ter. maxii	CBS 112496		EU707871	KF937242
Ter. micromaculata	CBS 124582	T	MH863390	MH874909
Ter. profusa	CBS 125007	T	KF901592	KF901916
Ter. profusa	CPC 12821		FJ493196	FJ493220
Ter. rubidae	CBS 124579	T	MH863388	MH874907
Ter. rubidae	MUCC659		EU300992	–
Ter. sieberi	CBS 144443	T	MH327816	MH327852
Ter. wingfieldii	CBS 112163	T	EU707896	–
Teratosphaericola pseudoafricana	CBS 114782	T	KF901737	KF902084
Terph. pseudoafricana	CBS 111168		KF901699	KF902045
Teratosphaeriopsis pseudoafricana	CBS 111171	T	KF901738	KF902085

[1]CAP = Culture Collection of Alan Phillips, housed at the Lab Bugworkers | M&B-BioISI | Tec Labs – Innovation Centre, Faculty of Sciences, University of Lisbon, Lisbon, Portugal, CBS = Culture Collection of the Westerdijk Fungal Biodiversity Institute, Utrecht, The Netherlands, CDP = Culture Collection of Diogo Pereira, housed at the Lab Bugworkers | M&B-BioISI | Tec Labs – Innovation Centre, Faculty of Sciences, University of Lisbon, Lisbon, Portugal, MUCC = Murdoch University Culture Collection, Murdoch, Australia.

[2]Status of the strains, T = ex-type, ET = ex-epitype.

[3]Newly generated sequences are in bold.

*ITS = internal transcribed spacers and intervening 5.8S nrDNA, LSU = partial large-subunit rRNA gene.

Appendix N. Coelomycetes presumptive identification details

Supplementary Table A.13 Presumptive identification of the coelomycetes isolates. The closest-hit genus and species represent the most probable identification considering the results obtained from GenBank after BLAST with ITS sequence.

Isolate	Closest-hit genus	Closest-hit species	Sequence coverage (%)	Sequence Identity (%)	Expected value	Sequence identity with type
CDP 467	*Colletotrichum*	*Colletotrichum karstii*	100	98.66	0	90
CDP 152	*Libertasomyces*	*Libertasomyces platani*	97	99.82	0	99.82
CDP 055	*Neosetophoma*	*Neosetophoma italica*	98	100	0	100
CDP 146	*Neosetophoma*	*Neosetophoma italica*	98	100	0	100
CDP 020	*Coniothyrium*	*Coniothyrium palmarum*	100	100	0	NA*
CDP 150	*Neosetophoma*	*Neosetophoma italica*	100	97.55	4×10^{-155}	97.55
CDP 139	–#	–	–	–	–	–
CDP 145	*Keissleriella*	*Keissleriella cladophila*	97	99.82	0	99.82
CDP 148	*Sclerostagonospora*	*Sclerostagonospora opuntiae*	100	98.95	0	NA
CDP 119	*Keissleriella*	*Keissleriella cladophila*	97	98.36	0	98.36
CDP 154	*Parastagonospora*	*Parastagonospora nodorum*	98	98.21	0	NA
CDP 217	*Paraconiothyrium*	*Paraconiothyrium variabile*	100	99.83	0	99.31
CDP 041	*Plenodomus*	*Plenodomus influorescens*	94	99.62	0	99.62
CDP 350	*Foliophoma*	*Foliophoma fallens*	98	100	0	NA
CDP 200	*Libertasomyces*	*Libertasomyces platani*	97	99.82	0	99.82
CDP 347	*Libertasomyces*	*Libertasomyces platani*	97	99.82	0	99.82
CDP 346	*Libertasomyces*	*Libertasomyces platani*	97	100	0	100
CDP 207	*Didymocyrtis*	*Didymocyrtis banksiae*	97	100	0	100
CDP 120	*Sclerostagonospora*	*Sclerostagonospora opuntiae*	100	98.77	0	NA
CDP 372	*Pseudoconiothyrium*	*Pseudoconiothyrium broussonetiae*	98	88.35	6×10^{-176}	88.35
CDP 378	*Stagonosporopsis*	*Stagonosporopsis cucurbitacearum*	98	99.80	0	NA
CDP 424	*Neosetophoma*	*Neosetophoma italica*	98	98.39	0	98.39
CDP 316	*Neosetophoma*	*Neosetophoma italica*	98	98.21	0	98.21
CDP 454	*Phaeosphaeria*	*Phaeosphaeria podocarpi*	100	99.61	0	99.61
CDP 317	*Didymocyrtis*	*Didymocyrtis brachylaenae*	96	99.28	0	99.28
CDP 371	*Keissleriella*	*Keissleriella cladophila*	97	100	0	100
CDP 270	*Paraconiothyrium*	*Paraconiothyrium brasiliense*	100	99.83	0	99.12
CDP 283	*Paraconiothyrium*	*Paraconiothyrium brasiliense*	100	99.66	0	98.95%
CDP 050	*Sclerostagonospora*	*Sclerostagonospora opuntiae*	100	99.12	0	NA
CDP 370	*Keissleriella*	*Keissleriella cladophila*	97	98	0	98
CDP 354	*Ascochyta*	*Ascochyta* sp.	100	98.27	0	-
CDP 375	*Keissleriella*	*Keissleriella cladophila*	97	98.36	0	98.36
CDP 248	*Coniothyrium*	*Coniothyrium palmarum*	92	100	0	NA
CDP 195	*Neosetophoma*	*Neosetophoma italica*	98	98.21	0	98.21
CDP 199	*Neosetophoma*	*Neosetophoma italica*	98	100	0	100
CDP 143	*Sclerostagonospora*	*Sclerostagonospora opuntiae*	100	99.77	0	NA

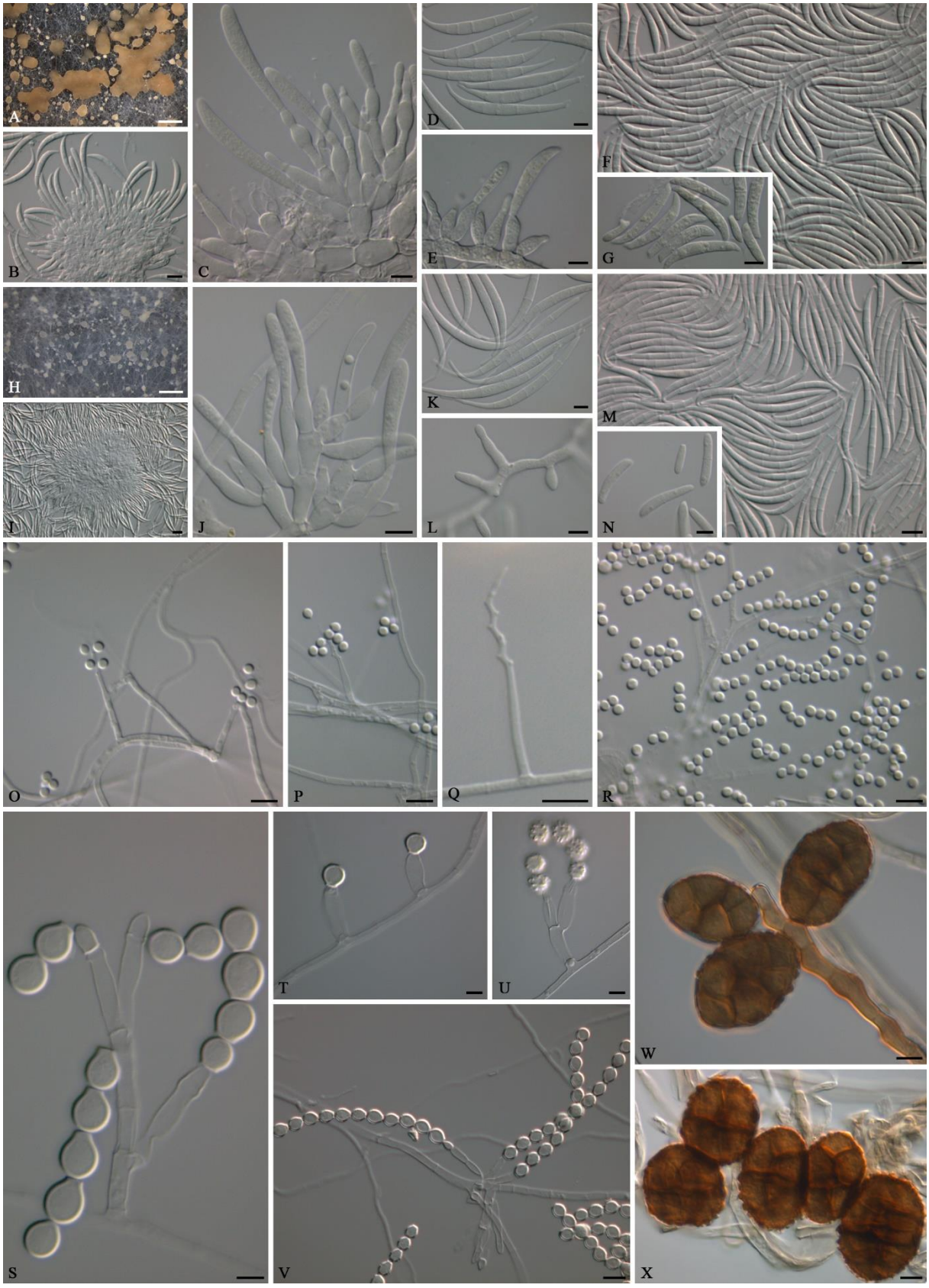
Supplementary Table A.13 Continued.

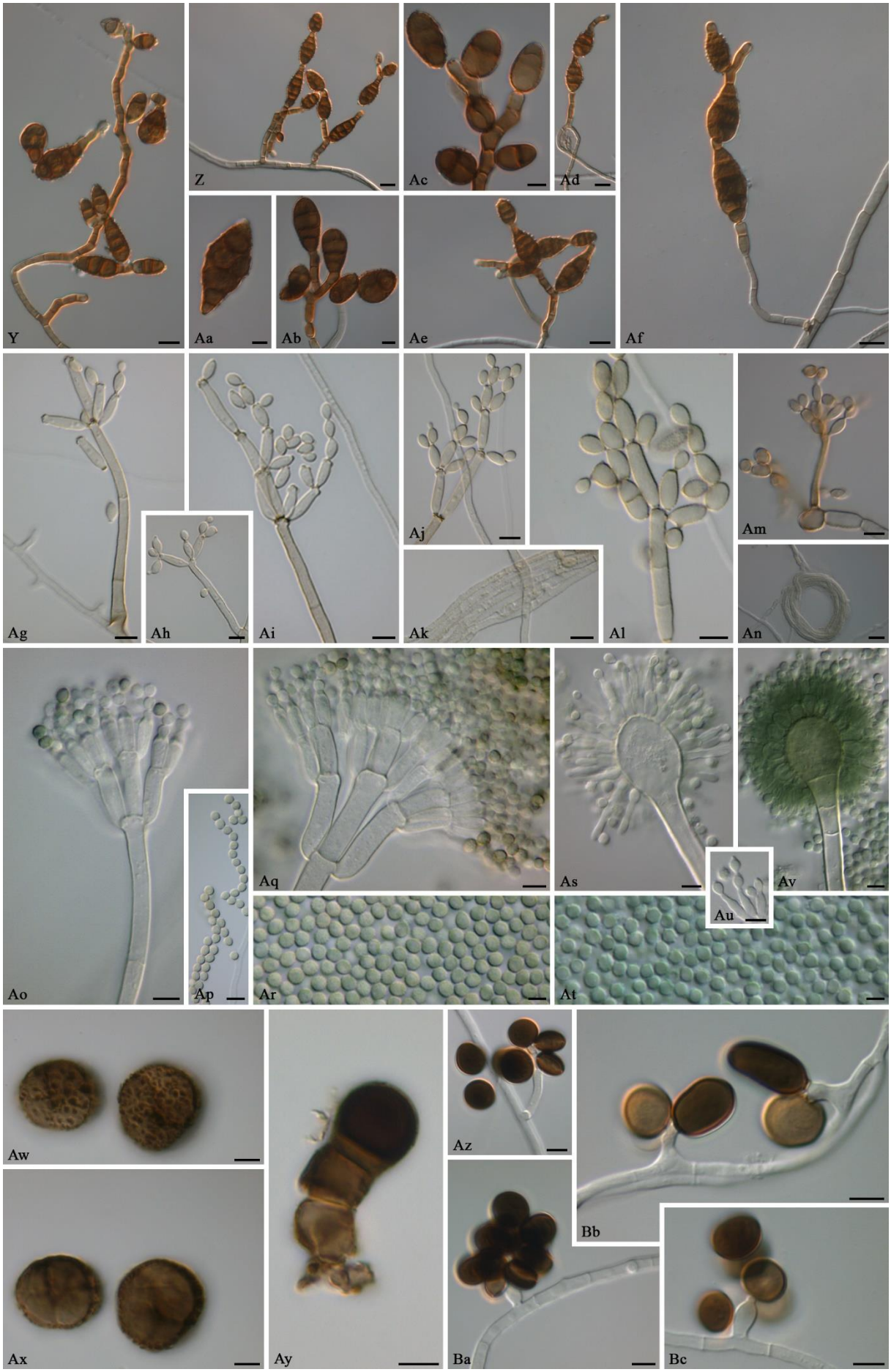
Isolate	Closest-hit genus	Closest-hit species	Sequence coverage (%)	Sequence Identity (%)	Expected value	Sequence identity with type
CDP 196	*Sclerostagonospora*	*Sclerostagonospora opuntiae*	100	99.12	0	NA
CDP 141	*Libertasomyces*	*Libertasomyces platani*	97	100	0	100
CDP 340	*Didymocyrtis*	*Didymocyrtis brachylaenae*	96	99.28	0	99.28
CDP 144	*Lophiostoma*	*Lophiostoma cynaroidis*	93.45	100	0	93.45
CDP 151	*Lophiostoma*	*Lophiostoma cynaroidis*	93.45	100	0	93.45

[*]NA, not available.

[#]–, not possible to predict a closest-hit.

Appendix O. Overview of morphological diversity

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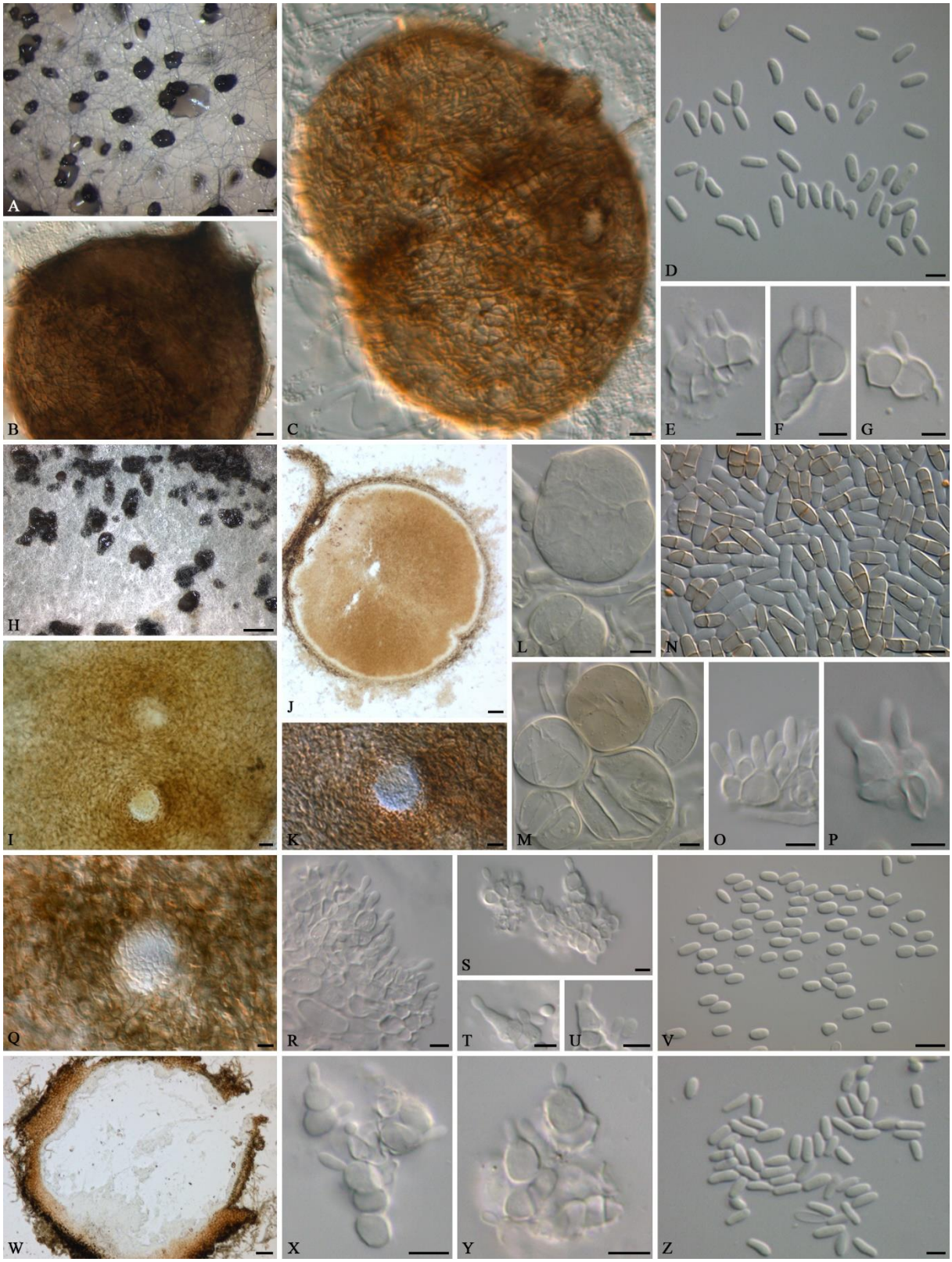
Supplementary Figure A.8 Overview of morphological diversity of palmicolous hyphomycetes from palms in Portugal. **A – N**. *Fusarium*. **O – R**. Hyphomycete genus 1. **S – V**. Hyphomycete genus 2. **W, X**. *Pithomyces*. **Y – Af**. *Alternaria*. **Ag – An**. *Cladosporium*. **Ao – Ar**. *Penicillium*. **As – Av**. *Aspergillus*. **Aw – Ay**. *Epicoccum*. **Az – Bc**. *Arthrinium*. **C, J, O – Q, S – W, Y, Z, Ab – Aj, Am, Ao, Aq, As, Au, Av, Ay – Bc**. Conidiophores and conidiogenous cells. **F, G, M, N, R, V, X, Z, Aa, Al, Ap, Ar, At, Aw, Ax, Ba**. Conidia. **A, H**. Sporodochia formed on palm leaflet pieces. **B, I**. Optical section of sporodochia. **C, J**. Macroconidiogenous cells. **D, F, K, M**. Macroconidia. **E, L**. Microconidiogenous cells. **G, N**. Microconidia. **Ak**. Mycelial ropes. **An**. Mycelial coils. Scale bars: A, H = 0.5 mm, B, I, F, M, V, Y, Z, Ad – Af = 10 μm, C – E, G, J – L, N, O – U, W, X, Aa – Ac, Ag – Bc = 5 μm.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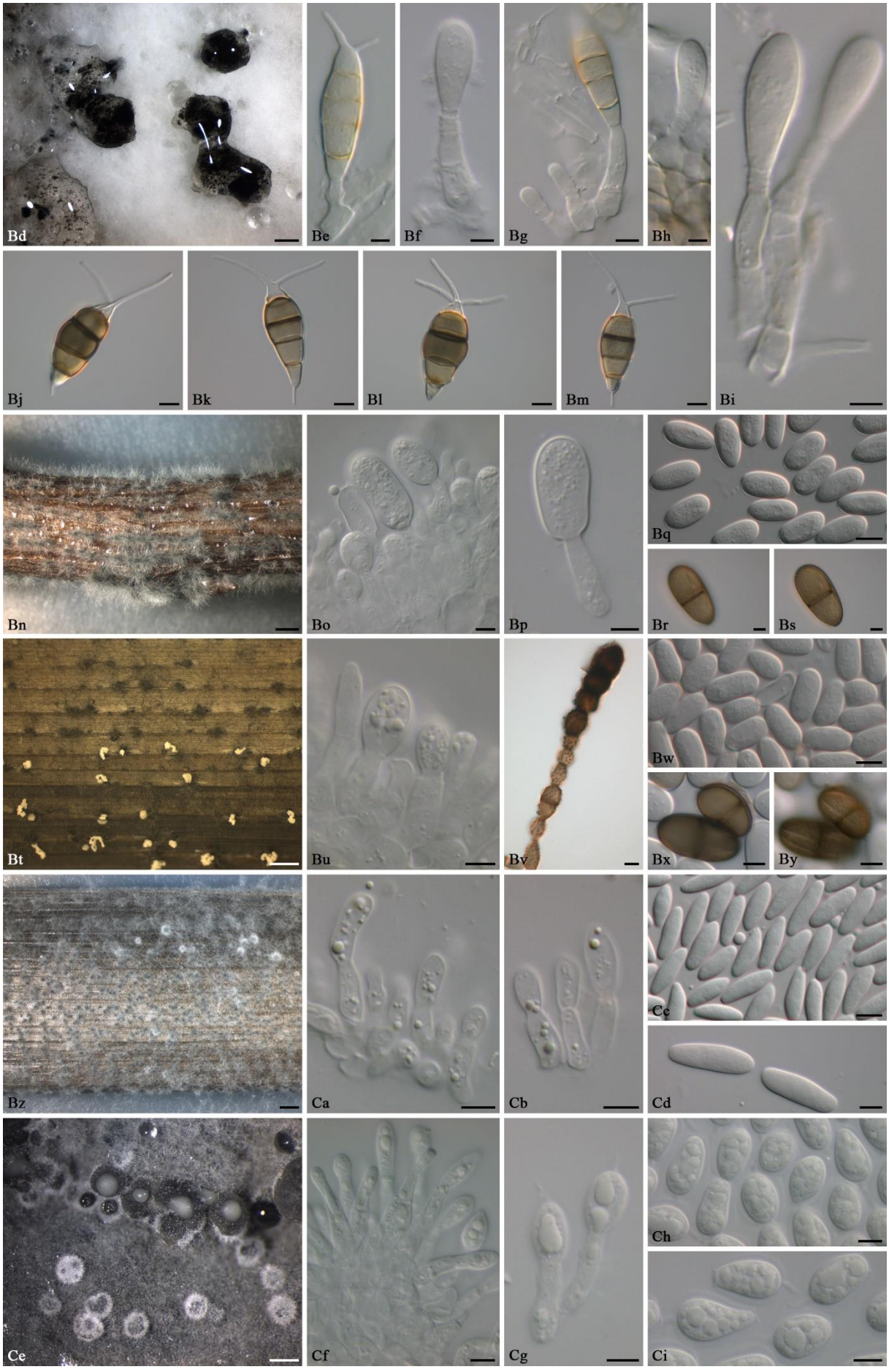
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Cf
Cg
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Ci

Supplementary Figure A.9 Overview of morphological diversity of palmicolous coelomycetes from palms in Portugal. **A – G**. *Phoma*. **H – P**. *Neosetophoma*. **Q – V**. *Libertasomyces*. **W – Z**. *Plenodomus*. **Aa – Ag**. *Sclerostagonospora*. **Ah – Al**. *Coniothyrium*. **Am – Ap**. *Septoria*. **Aq – Bc**. *Colletotrichum*. **Bd – Bm**. *Neopestalotiopsis*. **Bn – Bs**. *Diplodia*. **Bt – By**. *Neodeightonia*. **Bz – Cd**. *Neofusicoccum*. **Ce – Ci**. *Phyllosticta*. **A, H, Aa, Ah, Ai, Am, Bd, Ce**. Conidiomata formed on 1/2 PDA. **Bn**. Conidiomata formed on *Populus* sp. twig. **Bt, Bz**. Conidiomata formed on palm leaflet pieces. **Aq**. Acervulus formed on host tissue. **B, C**. Optical section of conidiomata. **J, W, Ac**. Vertical section of conidiomata. **Ab**. Section of conidioma wall. **I, K, Q**. Ostioles. **D, N, V, Z, Af, Ag, Aj, An, Aw, Bj – Bm, Bq – Bs, Bw – By, Cc, Cd, Ch, Ci**. Conidia. **E – G, O, P, R – U, X, Y, Ad, Ae, Ak, Ao, Ap, As – Av, Be – Bi, Bo, Bp, Bu, Ca, Cb, Cf, Cg**. Conidiogenous cells. **Ax**. Ascoma on host tissue. **Ay – Bb**. Asci. **Bc**. Ascospore. **Bv**. Chlamydospores. **L, M, Al**. Chlamydospore-like aerial hyphal swelling cells. **Aq – As**. Setae. Scale bars: H, Aa, Bd, Bt, Bz = 1 mm, Ah, Bn, Ce = 0.5 mm, Ai = 0.2 mm, A = 0.1 mm, Ac = 30 µm, C, I, J, W, Aq, Ax = 15 µm, B, Q, Ab, Ar = 10 µm, D – G, K – P, R – V, X – Z, Ad – Ag, Aj – Al, An – Ap, As – Aw, Ay – Bc, Be – Bm, Bo – Bs, Bu – By, Ca – Cd, Cf – Ci = 5 µm.