Hyperparasitic fungi on black mildews (Meliolales, Ascomycota): hidden diversity in the tropics

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CONTENTS

ABSTRACT

Meliolales (Sordariomycetes, Ascomycota) is a group of obligate plant parasitic microfungi mainly distributed in the tropics and subtropics. Meliolalean fungi are commonly known as "black mildews", as they form black, superficial hyphae on the surface of vegetative and reproductive organs of vascular plants. They are considered biotrophic parasites, and the infections caused by black mildews can lead to a decrease in the photosynthetic activity of plants, as well as to an increase in the temperature and respiration rate of their leaves.

Meliolales are frequently parasitized by hyperparasitic fungi, i.e., parasitic fungi that have parasitic hosts. These hyperparasites are all Ascomycota and belong mainly to the Dothideomycetes and Sordariomycetes. Although hyperparasites represent a megadiverse group, species were only described by morphology until 1980, and the systematic position of more than 60 % of known species is still unclear. In addition, there are no DNA reference sequences available in public databases for any of the species of hyperparasites of Meliolales, and no ecological studies have been done up to now.

Before this study, no exact number of hyperparasitic fungi growing on colonies of black mildews existed. Here, we present a checklist including 189 species of fungi known to be hyperparasitic on Meliolales, but the number of existing species is likely to be even higher. The elaboration of this species checklist laid the foundations for this investigation, as it helped to understand the present state of knowledge of hyperparasitic fungi on Meliolales worldwide.

For the present study, fresh specimens of leaves infected with colonies of Meliolales and hyperparasites were opportunistically collected at 32 collection sites in Western Panama and Benin, West Africa, in 2020 and 2022, respectively. In total, 100 samples of plant specimens infected with black mildews were collected, of which 58 samples were parasitized by hyperparasitic fungi. 31 species and morphospecies of hyperparasitic fungi were identified. In addition, 35 historical specimens, including 12 type specimens, were examined for the present work.

DNA of hyperparasitic fungi was isolated directly from conidia, synnemata, apothecia, perithecia or pseudothecia of fresh and dried specimens. The main challenges faced by scientists in doing molecular studies of hyperparasitic fungi are related to the fact that the

hyperparasitic fungi are intermingled with tissues of the meliolalean hosts and other organisms present in a given sample. This makes the isolation of DNA exclusively from the hyperparasite difficult. Moreover, hyperparasitic fungi on Meliolales are biotrophs and cannot be grown axenically. The hosts themselves are also biotrophic, further complicating DNA isolation from either partner. These factors have contributed to a lack of reference sequences in public databases. After more than 100 attempts, DNA of 20 specimens of hyperparasitic fungi, representing seven species, has been isolated in the context of the present investigation. Three partial nuclear gene regions were amplified and sequenced: nrLSU, nrSSU and nrITS. The datasets were assembled for phylogenetic analyses applying Maximum Likelihood (ML) and Bayesian inference (BI) methods. DNA sequences of hyperparasitic fungi on Meliolales were generated for the first time in the context of the present investigation.

Hyperparasitic fungi on Meliolales do not represent a single systematic group, but a polyphyletic ecological guild of fungi. Because of this huge diversity, only the systematics of species of perithecioid hyperparasites, as well as of the species of the genera *Atractilina* and *Spiropes* known to be hyperparasitic on black mildews was discussed in this thesis, as they represented the most common groups of fungi found in Benin and Panama. The results indicated, for example, the systematic position of *Dimerosporiella cephalosporii* and *Paranectriella minuta* in the Sordariomycetes and Dothideomycetes, respectively. In addition, the first record of a hyperparasitic fungus of black mildews in the Lecanoromycetes, namely *Calloriopsis herpotricha*, is reported here. The systematics of *Atractilina parasitica* and of some species of *Spiropes* is also discussed here.

In the context of the present investigation, four species new to science were described. They are presented with detailed descriptions, photos and scientific illustrations. Taxonomic studies of this thesis also generated seven new synonyms, nine new records for Benin, seven for Panama, one for Africa and two for mainland America, as well as the confirmation of one anamorph-teleomorph connection by molecular sequence data.

The ecology of hyperparasitic fungi on Meliolales is complex and far from being completely understood. The hypothesis of host specificity between hyperparasitic fungi, their meliolalean hosts and their plant hosts was tested for the first time, through a tritrophic network analysis. Results indicate that hyperparasites of Meliolales are generalists concerning genera of Meliolales, but apparently specialists at the level of order. In addition, hyperparasitic fungi tend to be found alongside their meliolalean hosts, suggesting a pantropical distribution.

The overall objective of this thesis is to contribute new information on the diversity, ecology and systematics of hyperparasitic fungi on colonies of Meliolales in the tropics.

ZUSAMMENFASSUNG

Eines der interessantesten Phänomene in der Natur ist die Tatsache, dass selbst Parasiten ihre eigenen Parasiten haben. Dies zeigt, dass in Ökosystemen die Interaktionen zwischen den Arten durch ein komplexes multitrophisches Netzwerk dargestellt werden, in dem alle Arten auf die eine oder andere Weise miteinander interagieren. Dieses Phänomen, bei dem ein Parasit von einem anderen parasitären Organismus parasitiert wird, wird Hyperparasitismus genannt. Hyperparasitismus kommt in der Natur häufig vor und ist von größter Bedeutung für die Dynamik der Interaktion zwischen Wirten und ihren Parasiten, für die Erhöhung der Komplexität von Nahrungsnetzen und für die Regulierung von Populationsgrößen. Dieses Phänomen ist für parasitoide Insekten, parasitische Blütenpflanzen und krankheitsverursachende Viren in Protozoen gut dokumentiert. Der durch Pilze verursachte Hyperparasitismus ist jedoch kaum untersucht worden.

Das Studiensystem für die vorliegende Untersuchung bestand aus hyperparasitischen Pilzen, die auf Kolonien von Meliolales (Sordariomycetes, Ascomycota) wachsen, einer Gruppe obligat pflanzenparasitärer Mikropilze aus den Tropen und Subtropen. Meliolales-Arten sind gemeinhin als "schwarze Mehltaupilze" bekannt, da sie schwarze, oberflächliche Hyphen auf der Oberfläche von vegetativen und reproduktiven Organen von Gefäßpflanzen bilden. Sie gelten als biotrophe Parasiten, und die von den schwarzen Schimmelpilzen verursachten Infektionen können zu einer Verringerung der photosynthetischen Aktivität der Pflanzen sowie zu einem Anstieg der Temperatur und der Atmungsrate ihrer Blätter führen. Meliolales gelten jedoch nicht als aggressive Parasiten wie etwa Rostpilze.

Meliolales werden häufig von hyperparasitischen Pilzen parasitiert, so dass es oft unmöglich ist, den Wirt der Meliolales zu identifizieren und zu isolieren. Diese Hyperparasiten sind alle Ascomycota und gehören hauptsächlich zu den Dothideomycetes und Sordariomycetes. Obwohl sie eine sehr artenreiche Gruppe darstellen, wurde die Erforschung der hyperparasitischen Pilze auf Meliolales abrupt eingestellt, bevor molekulare Techniken in der Pilztaxonomie weit verbreitet waren. Infolgedessen wurden die Arten nur morphologisch beschrieben, und die heutige systematische Stellung von mehr als 60 % der bekannten Arten ist immer noch unklar. Darüber hinaus wurden Aspekte wie ökologische Verbreitungsmuster und Wirtsspezifität bis zur vorliegenden Untersuchung nicht erörtert. Das übergeordnete Ziel

dieser Arbeit war es, neue Informationen über die Vielfalt, Ökologie und Systematik hyperparasitischer Pilze auf Kolonien von Meliolales in den Tropen zu liefern.

Vor dieser Studie gab es keine genaue Zahl der hyperparasitären Pilze, die auf Kolonien von Schwarzem Mehltau wachsen. Wir geben hier 189 Pilzarten an, von denen bekannt ist, dass sie auf Meliolales hyperparasitisch sind, aber diese Zahl ist wahrscheinlich noch höher. Die Ausarbeitung dieser Artenliste war für diese Untersuchung von entscheidender Bedeutung, da sie dazu beitrug, den derzeitigen Kenntnisstand über hyperparasitäre Pilze auf Meliolales weltweit zu verstehen, und somit die Grundlage für dieses Forschungsprojekt bildete.

In der vorliegenden Studie wurden frische Proben von Blättern, die mit Kolonien von Meliolales und Hyperparasiten infiziert waren, an 32 Sammelstellen in Benin, Westafrika und Westpanama in den Jahren 2020 und 2022 opportunistisch gesammelt. Insgesamt wurden 100 Proben von mit schwarzem Mehltau infizierten Pflanzen gesammelt, von denen 58 Proben von hyperparasitischen Pilzen parasitiert waren. Es wurden 31 Arten und Morphospezies von hyperparasitären Pilzen identifiziert. Darüber hinaus wurden 35 historische Exemplare, darunter 12 Typusarten, für die vorliegende Arbeit untersucht. Die häufigste Art in Panama war *Spiropes melanoplaca*, während *Atractilina parasitica* die häufigste Art in Benin war. Beide Arten wurden wiederholt an verschiedenen Sammelstellen und zu unterschiedlichen Zeitpunkten gesammelt. Der perithezioide Hyperparasit, *Dimerosporiella cephalosporii*, war in beiden Ländern die häufigste Art. Der Artenreichtum an hyperparasitischen Pilzen auf Meliolales war in Panama höher als in Benin.

Im Rahmen der vorliegenden Untersuchung wurden vier neue Arten beschrieben, die für die Wissenschaft neu sind. Sie werden hier mit detaillierten Beschreibungen, Fotos und wissenschaftlichen Illustrationen vorgestellt. Die neuen Arten sind: *Paranectria longiappendiculata*, *Spiropes angylocalycis*, *Spiropes carpolobiae* und *Spiropes croissantiformis*. Die taxonomischen Untersuchungen im Rahmen dieser Arbeit erbrachten auch sieben neue Synonyme für die Gattung *Spiropes*. Darüber hinaus wurden neun Arten für Benin, sieben für Panama, eine für Afrika und zwei für das amerikanische Festland neu beschrieben.

Die DNA von hyperparasitären Pilzen wurde direkt aus Konidien, Synnemata, Apothecien, Perithecien oder Pseudothecien frischer und getrockneter Exemplare isoliert. Nach mehr als 100 Versuchen wurde im Rahmen der vorliegenden Untersuchung zum ersten Mal DNA von

20 Exemplaren hyperparasitärer Pilze isoliert, die 7 Arten repräsentieren. Drei partielle Kerngenregionen wurden amplifiziert und sequenziert: nrLSU, nrSSU und nrITS. Die Datensätze wurden für phylogenetische Analysen unter Anwendung von Maximum-Likelihood- (ML) und Bayes'schen Inferenzmethoden (BI) zusammengestellt. Im Rahmen der vorliegenden Untersuchung wurden erstmals DNA-Sequenzen von hyperparasitischen Pilzen auf Meliolales erstellt.

Hyperparasitische Pilze auf Meliolales stellen keine systematische Gruppe dar, sondern sind eine polyphyletische ökologische Gilde von Pilzen. Dies zeigt sich an der großen Bandbreite an Fortpflanzungsstrukturen und Morphologien, die diese Pilze aufweisen. Aufgrund dieser enormen Vielfalt wurde in dieser Arbeit nur die Systematik der perithecioiden Hyperparasiten sowie der Arten der Gattungen *Atractilina* und *Spiropes*, die als Hyperparasiten des schwarzen Mehltaus bekannt sind, behandelt. Dies waren die in Benin und Panama am häufigsten vorkommenden Pilzgruppen. Die systematischen Studien wurden hauptsächlich durch morphologische Untersuchungen mittels Licht- und Rasterelektronenmikroskopie durchgeführt. So wurde beispielsweise die systematische Stellung von *Dimerosporiella cephalosporii* bei den Hypocreales und von *Paranectriella minuta* bei den Dothideomycetes sowohl durch morphologische als auch durch DNA-Sequenzdaten bestätigt. *Calloriopsis herpotricha* hingegen wird als erster Nachweis eines hyperparasitischen Pilzes auf Meliolales in den Lecanoromycetes erkannt.

Einer der wichtigsten Beiträge dieser Arbeit ist die Bestätigung einer anamorph-teleomorphen Verbindung zwischen *Atractilina parasitica* und *Malacaria meliolicola* durch molekulare Sequenzdaten. In Benin wurden beide Arten gemeinsam auf Blättern von *Coffea arabica* gefunden. Die erstmals gewonnenen nrLSU-DNA-Sequenzen von *A. parasitica* wiesen einen Ähnlichkeitsgrad von 98 % mit den neu generierten Sequenzen von *M. meliolicola* auf. Damit wurden die systematische Stellung von *A. parasitica* innerhalb der Dothideomyceten und die anamorph-teleomorphe Verbindung zwischen diesen beiden Arten bestätigt. *Atractilina parasitica* könnte auch zur Ordnung Pleosporales s.l. gehören, aber um diese systematische Hypothese zu bestätigen und die Stellung von *A. parasitica* auf Familienebene zu bestimmen, ist die Verwendung mehrerer Loci erforderlich. Bei den *Spiropes*-Arten war es möglich, DNA-Sequenzdaten von zwei Arten zu erhalten. Diese nrITS-DNA-Sequenzen deuten darauf hin, dass die Gattung *Spiropes* möglicherweise polyphyletisch ist und zu den Leotiomyceten gehört.

Die im Rahmen der vorliegenden Untersuchung erzielten Ergebnisse stellen erste Beiträge zur Erforschung der Systematik der hyperparasitären Pilze auf Meliolales dar. Ein breiteres Taxon-Sampling, eine Neubewertung bestehender Gattungs- und Artkonzepte und die Analyse zusätzlicher Genregionen werden notwendig sein, um die Auflösung der Untersuchung dieser Organismen in zukünftigen Studien zu verbessern.

Die molekulare Untersuchung von hyperparasitären Pilzen ist nach wie vor eine Herausforderung. Da sie zu verschiedenen systematischen Abstammungslinien gehören und unterschiedliche Morphologien aufweisen, wurden bisher keine spezifischen molekularen Methoden zu ihrer Untersuchung entwickelt. Die größte Herausforderung für die Wissenschaftler bei der Durchführung molekularer Studien ist die Tatsache, dass die hyperparasitären Pilze oft mit Geweben der meliolalischen Wirte und anderen Organismen in einer bestimmten Probe vermischt sind. Dies macht die Isolierung von DNA aus dem Hyperparasiten schwierig. Außerdem sind hyperparasitäre Pilze auf Meliolales biotroph und können nicht axenisch kultiviert werden. Die Wirte selbst sind ebenfalls biotroph, was die DNA-Isolierung von beiden Partnern weiter erschwert. Diese Faktoren haben zu einem Mangel an Referenzsequenzen in öffentlichen Datenbanken geführt. Vor dieser Untersuchung gab es keine DNA-Referenzsequenzen von hyperparasitischen Pilzen auf Meliolales.

Alle Versuche, die Exemplare aus Benin und Panama auf künstlichen Nährböden zu kultivieren, schlugen fehl. Die Unfähigkeit der hyperparasitären Pilze, auf künstlichen Nährböden zu wachsen, die Tatsache, dass sie im Freiland nur zusammen mit den Meliolen-Wirten wachsen, und die Ergebnisse der mikroskopischen Analysen, die in dieser Untersuchung durchgeführt wurden, deuten jedoch darauf hin, dass diese Pilze obligate Parasiten sind. Die morphologischen Analysen der gesammelten Exemplare mittels Licht- und Rasterelektronenmikroskopie zeigten, dass diese Pilze einen engen Kontakt mit den Hyphen und den Sporen der Meliolales herstellen, ohne dass Haustorien vorhanden sind.

Im Rahmen der vorliegenden Untersuchung wurde die Hypothese der Wirtsspezifität zwischen hyperparasitären Pilzen, ihren Meliolales-Wirten und ihren pflanzlichen Wirten zum ersten Mal durch eine Analyse des tri-trophischen Netzwerks geprüft. Dieses Netzwerk zeigte, dass Arten hyperparasitärer Pilze Generalisten in Bezug auf Gattungen der Meliolales sind, d. h. das Wirtsspektrum der meisten Gattungen hyperparasitärer Pilze umfasst mehrere Arten einer oder mehrerer Gattungen von schwarzem Mehltau. Hyperparasitäre Pilze können auch auf der Ebene der Ordnung Spezialisten sein, da viele Arten nur auf Meliolales-Wirten wachsen.

Letzteres ist jedoch mit Vorsicht zu genießen, da es an Daten mangelt und die Hyperparasiten der Meliolales und anderer pflanzenparasitischen Pilze nicht ausreichend untersucht wurden. So sind einige Arten im Netz nur durch einen einzigen Beleg vertreten, was zu der irrigen Schlussfolgerung führt, dass es sich um eine sehr spezifische Art handelt. Andere Arten, wie z. B. *Atractilina parasitica*, wurden mehr als 60 Mal auf verschiedenen Gattungen und Arten der Meliolales nachgewiesen. Der Aufbau multitrophischer ökologischer Netze ist eine schwierige Aufgabe, vor allem in schlecht untersuchten und sehr vielfältigen Systemen, wie es bei hyperparasitären Pilzen und schwarzem Mehltau der Fall ist. Der Aufwand für Probenahmen muss erhöht werden, und es sollten Daten aus mehr Ländern und Wirtspilzen einbezogen werden, um künftige Analysen der Interaktionen dieser Arten zu verbessern.

Hyperparasitäre Pilze sind auf das Vorhandensein ihrer Wirte angewiesen. Da die Artenvielfalt der Meliolales in den Tropen höher ist als in außertropischen Breiten und, wie bereits erwähnt, hyperparasitische Pilze auf Meliolales-Wirte beschränkt zu sein scheinen, ist zu erwarten, dass in den Tropen auch eine große Vielfalt an Hyperparasiten zu finden ist. Tatsächlich zeigen die in dieser Arbeit vorgestellten Ergebnisse eine überraschend hohe Diversität von Hyperparasiten in Benin und Panama, basierend auf nur wenigen Monaten Feldarbeit. Weitere Probenahmen werden sicherlich unser Wissen über hyperparasitäre Pilze auf Meliolales erweitern. Darüber hinaus scheinen hyperparasitäre Pilzarten eine pantropische Verbreitung zu haben, da sie in den Neotropen, Afrotropen und indomalayischen Ökoregionen gefunden wurden. *Dimerosporiella cephalosporii* und *Spiropes melanoplaca* zum Beispiel sind zwei häufige Arten von Hyperparasiten von Schwarzen Mehltaupilzen, die wiederholt in Benin und Panama gefunden wurden.

Die vorliegende Untersuchung stellt einen Fortschritt im Verständnis der Systematik, Taxonomie und Ökologie der hyperparasitären Pilze der Meliolales dar und verdeutlicht deren enorme Vielfalt in den Tropen sowie das Potenzial für weitere Erforschung dieser Organismen.

LIST OF ORIGINAL PUBLICATIONS AND MANUSCRIPTS

This thesis is based on three peer-reviewed publications and one manuscript, which are cited in the following sections as follows:

Bermúdez-Cova et al. (2022)
Bermúdez-Cova MA, Cruz-Laufer AJ, Piepenbring M. 2022. Hyperparasitic fungi on black mildews (Meliolales, Ascomycota): Hidden fungal diversity in the tropics. Frontiers in Fungal Biology 3:885279. https://doi.org/10.3389/ffunb.2022.885279

Bermúdez-Cova et al. (2023a)
Bermúdez-Cova MA, Haelewaters D, de Bekker C, Piepenbring M, Schoutteten N, Quandt A. 2023a. Hyperparasitic fungi—definitions, diversity, ecology, and research. Available as preprint on: 10.22541/au.168787020.07281183/v1. **Awaiting publication of the book.**

Bermúdez-Cova et al. (2023b)
Bermúdez-Cova MA, Krauß A, Sanjur A *et al.* 2023b. Diversity of hyperparasitic fungi on Meliolales (Sordariomycetes, Ascomycota): new species, records, and molecular data from Benin and Panama. Mycological Progress 22, 65. https://doi.org/10.1007/s11557-023-01913-5.

Manuscript 1
Bermúdez-Cova MA, Hofmann TA, Yorou NS, Piepenbring M. 2023. Systematic revision of species of *Atractilina* and *Spiropes* hyperparasitic on Meliolales in the tropics. **Status: submitted.**

INTRODUCTION

Epifoliar fungi

Epifoliar fungi are a group of ascomycetous fungi that complete their entire life cycle on the surface of living plants (Gilbert et al. 2007, Schoch et al. 2009, Zeng et al. 2020, Marasinghe et al. 2023). They share morphological adaptations such as melanin pigmentation (Reynolds and Gilbert 2006). The major orders of epifoliar fungi are Asterinales, Meliolales, Microthyriales and Zeloasperisporiales. They live as saprobes, obligate parasites or commensals on plants (Marasinghe et al. 2022). The largest order of epifoliar fungi is **Meliolales** (Figure 1; Zeng et al. 2017, Bánki et al. 2023).
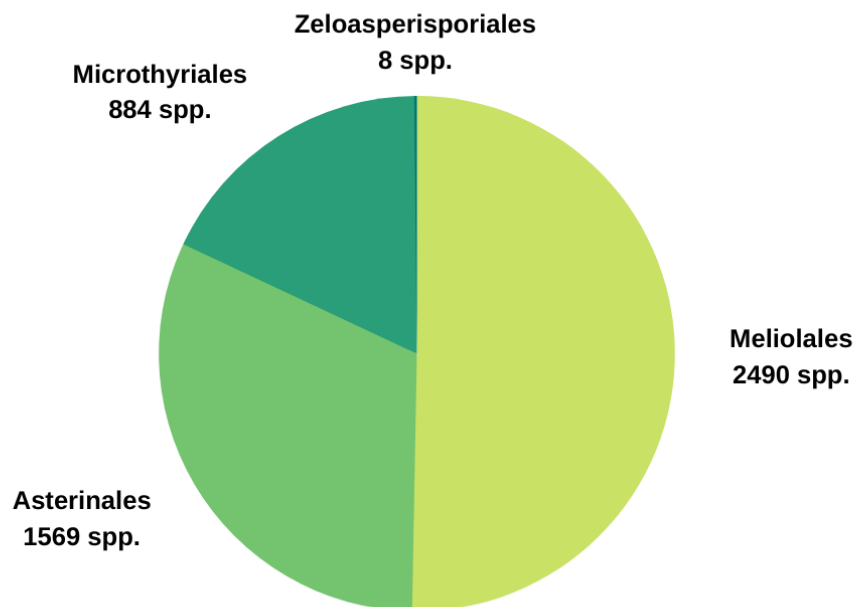
Figure 1 | Diagram reflecting the number of known species in the four major orders of epifoliar fungi.

Meliolales

Meliolales (Sordariomycetes, Ascomycota), commonly known as **"black mildews"**, is an order of biotrophic, obligate plant parasitic microfungi in the tropics and subtropics (Schmiedeknecht 1995, Hosagoudar 2006, Araúz and Piepenbring 2012). The order comprises nine genera distributed in two families, namely Armatellaceae and Meliolaceae, with *Armatella* and *Meliola* being the most species-rich genera of each family, respectively (Hosagoudar 2003, Jayawardena et al. 2020, Bánki et al. 2023).

Black mildews form dark, thick-walled, branched, superficial hyphae on the leaves, petioles, twigs and fruits of vascular plants (Fig. 2; Piepenbring et al. 2011, Hongsanan et al. 2015, Zeng et al. 2017, 2020). The lateral branches of hyphae are called hyphopodia (Fig. 3; Hongsanan et al 2015). Species of Meliolaceae form capitate and mucronate hyphopodia (Piepenbring 2015). Capitate hyphopodia are formed by a foot cell and a globose/lobate terminal cell. This terminal cell acts as an appressorium. A peg formed by the appressorium penetrates the leaf surface and forms a haustorium inside the epidermal host cell to absorb nutrients (Hansford 1961, Piepenbring et al. 2011). Other lateral branches, the phialides, or mucronate hyphopodia, consist of a single, bottle-shaped cell, which can form small spores at the tips. These spores can function as conidia or spermatia, but they have been poorly studied (Goos 1974, Piepenbring et al. 2011). Species of the family Armatellaceae form stellate hyphopodia and lack phialides (Fig. 3; Hongsanan et al. 2015). Meliolalean fungi present globose perithecia containing asci with dark brown, aseptate to 1-septate to transversely multiseptate ascospores. Most species also present long setae attached to superficial hyphae and/or perithecia (Hosagoudar and Thomas 2013).
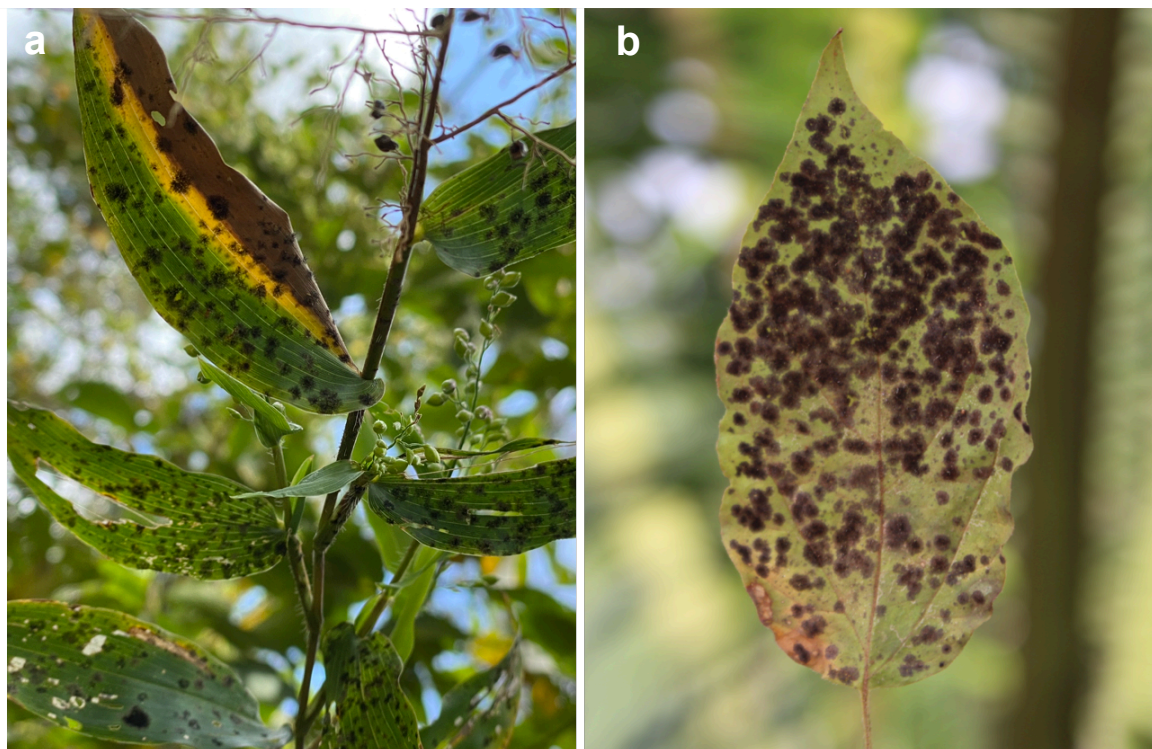

Figure 2 | Colonies of Meliolales (black spots). a On living leaves of *Olyra latifolia* (Poaceae) in Panama; **b** On a living leaf of *Clerodendrum capitatum* (Lamiaceae) in Benin, West Africa.

Infections by species of Meliolales result in a reduction of chlorophyll, starch, sugar, proteins and amino acids in the affected areas of the plant host (Hosagoudar et al. 1997, Old et al. 2003, Rodríguez Justavino and Piepenbring 2007). Respiration rates and the temperature of the infected areas may increase due to the lesions and the black color, and photosynthetic activity may also be reduced (Hosagoudar et al. 1997, Hongsanan et al. 2014). These infections result in a "dirty" appearance of the hosts, thus, reducing their economic value as ornamental plants (Hosagoudar et al. 1997). However, meliolalean fungi are not known to cause significant damage to crops (Hosagoudar 2006).
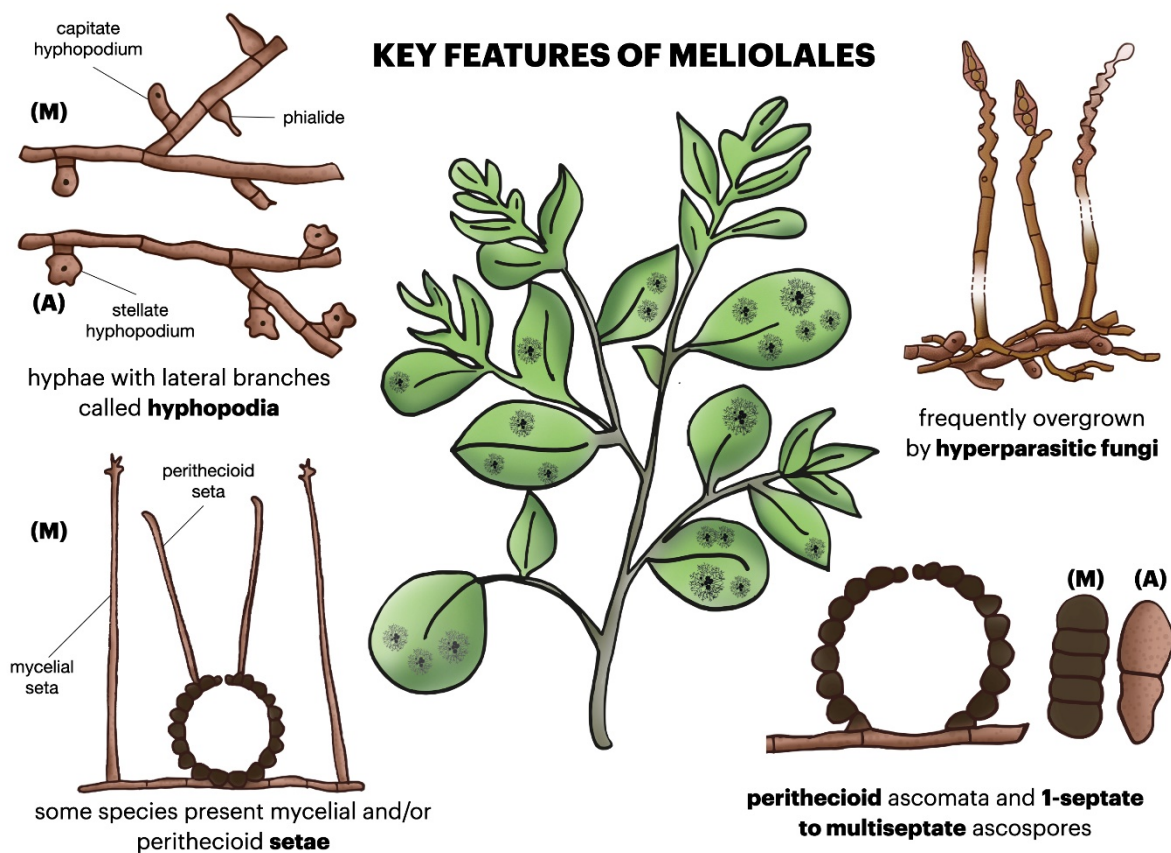
Figure 3 | **Generalized key features of species of Meliolales.** Some characteristics apply only to species of the family Armatellaceae **(A)** and some to species of the family Meliolaceae **(M).**

Along with species of Erysiphaceae, Phyllachorales, Pucciniales and Ustilaginales, Meliolales are plant parasitic fungal hosts that are frequently infected by hyperparasitic fungi (Hawksworth 1981, Gams et al. 2004).

Hyperparasitic fungi

Hyperparasitic fungi, i.e., fungi parasitic on other parasitic hosts, can be found across the fungal tree of life, from Cryptomycota to Basidiomycota and Ascomycota, as well as in fungus-like organisms such as Oomycota (Jeffries 1985, Lutz et al. 2004, Gleason et al. 2012). Hyperparasitic fungi may be biotrophic or necrotrophic parasites (Boosalis 1964, Barnett and Binder 1973, Jeffries 1995, Benjamin et al. 2004, Sun et al. 2019). Necrotrophic parasites kill their hosts, while biotrophic parasites take nutrients from the living cells of their hosts (Jeffries 1995, Moore et al. 2020). Hyperparasitic fungi may shape the dynamics of the interaction between the plant host and the fungal host, increase the complexity of the food webs and play a significant role in regulating population sizes (Gleason et al. 2014, Sandhu et al. 2021).

The present study focuses specifically on hyperparasitic fungi that grow on colonies of black mildews. For an updated revision of hyperparasitic fungi on other fungal and non-fungal hosts, see **Publication 2 (Bermúdez-Cova et al. 2023a)**.

Hyperparasitic fungi on Meliolales

Diversity and systematics

Information about fungal hyperparasites on colonies of Meliolales is scattered throughout literature and, before this study, no exact number of known species existed. **Publication 1 (Bermúdez-Cova et al. 2022)** represents the first review on hyperparasites of Meliolales, and a checklist of hyperparasites on black mildews known worldwide was made based on databases, herbarium specimens and literature. In total, **189** species of hyperparasitic fungi are known to occur on colonies of Meliolales worldwide. The most important analyses derived from this checklist regarding the history of description, distribution, ecology and systematics of hyperparasitic fungi are discussed in the upcoming sections.

Hyperparasitic fungi of black mildews are all ascomycetes and belong to several systematic groups, mainly to the Dothideomycetes and Sordariomycetes (Ciferri 1955, Deighton and Pirozynski 1972, Gams et al. 2004). Most species have been described based on morphology before the widespread use of molecular techniques in fungal taxonomy. Therefore, the modern systematic position of at least 60 % of the species of hyperparasitic fungi is still unknown, and DNA sequences of known species are completely lacking in public databases (**Bermúdez-Cova et al. 2022**).

Hyperparasites of black mildews form an ecological guild of organisms that share a common lifestyle, rather than a single systematic group. Thus, we propose a morphological classification of hyperparasitic fungi on Meliolales based on their spore-producing structures. This classification results in six morphological groups (Fig. 4): apothecioid fungi, catathecioid fungi, dematiaceous hyphomycetes, moniliaceous hyphomycetes, perithecioid hyperparasites and pycnidioid fungi. These morphological groups are discussed in detail in **Bermúdez-Cova et al. (2002).** This classification is purely artificial, but it is useful to show the great diversity of reproductive structures and systematic groups of fungi parasitic on Meliolales.
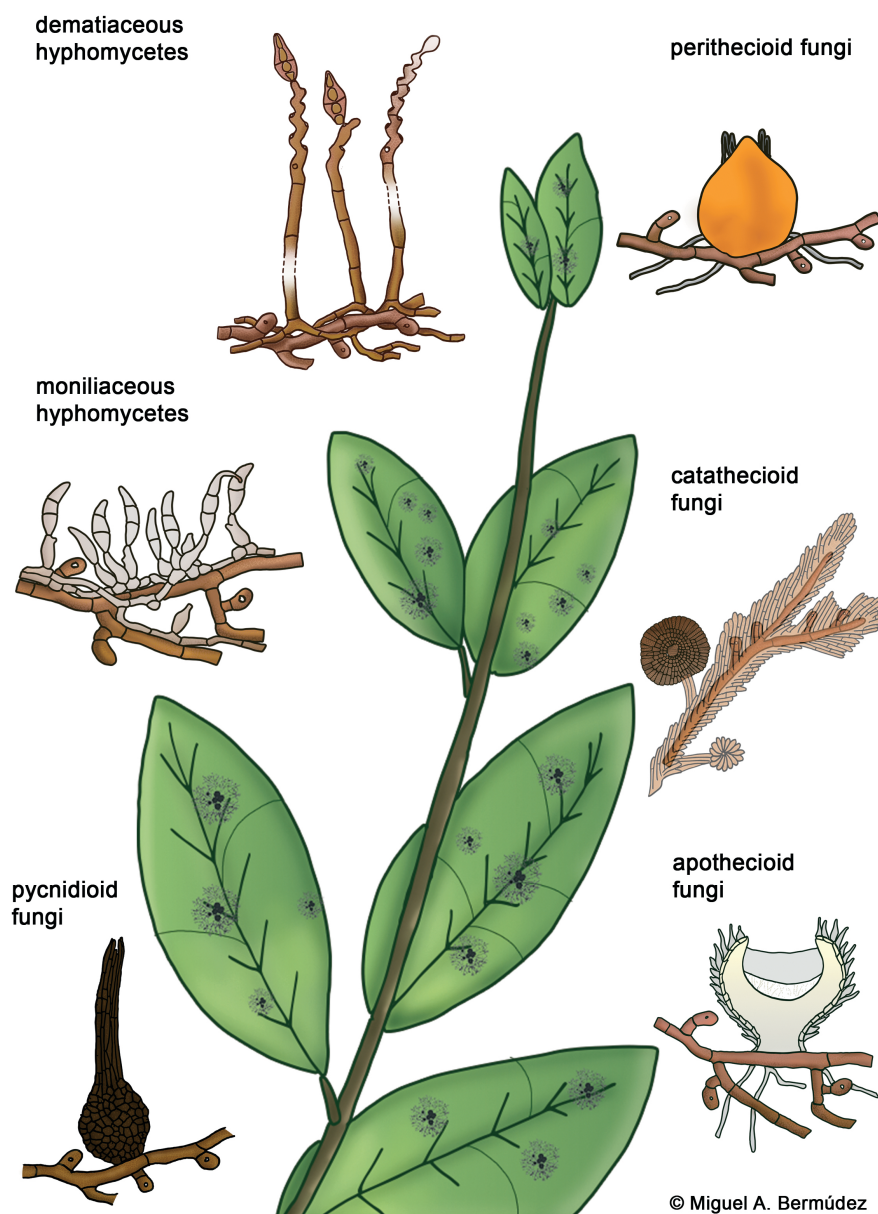

Figure 4 | Morphological groups of hyperparasitic fungi that grow on colonies of black mildews (Meliolales, Ascomycota). The figure was taken from Bermúdez-Cova et al. (2022).

Ecology

Hyperparasitic fungi that grow on colonies of Meliolales are most probably obligate biotrophs, as they have been found in the field only growing together with the fungal host, and there are no records of cultivation on artificial media (**Bermúdez-Cova et al. 2022**). Based on morphological and physiological observations only, nutrients are possibly transferred via an interface of close cell-to-cell contact. However, no ultrastructural studies of the interaction between hyperparasitic fungi and black mildews have been done in the past (Jeffries 1995, **Bermúdez-Cova et al. 2022**). Although hyperparasitic fungi on Meliolales seem to be restricted to meliolalean hosts (**Bermúdez-Cova et al. 2022**), no studies on host-specificity have been done in the past.

Parasitic fungi can easily overgrow the entire colonies of the black mildews and prevent the meliolalean fungus from producing spores and ascomata (Stevens 1918, Toro 1952). Hyperparasites also modify some vegetative structures of Meliolales, such as the density and branching of hyphae, the number, shape or size of hyphopodia, and the presence, number, disposition, size, and shape of setae (Ciferri 1955).

Hyperparasitic fungi on Meliolales in Benin, West Africa and Panama

According to the most recent checklists of the funga from Benin (Piepenbring et al. 2020) and Panama (Hofmann and Piepenbring 2021), there are currently three species of Meliolales known for Benin and 105 for Panama. Prior to the present investigation, there were no records of hyperparasitic fungi on Meliolales for either of these countries. **Publication 3** (**Bermúdez-Cova et al. 2023b**) and **Manuscript 1** include four new species of hyperparasitic fungi on Meliolales, as well as several new reports for Benin and Panama, with emphasis on their taxonomy, systematics and ecology, and also including DNA sequences generated for the first time in the context of the present investigation.

Hyperparasitic fungi on Meliolales represent a hyperdiverse but understudied ecological group of fungi in the tropics. The overall objective of this thesis is to contribute new information on the diversity, ecology and systematics of hyperparasitic fungi on colonies of Meliolales in the tropics, by using Benin, West Africa, and Panama as study areas.

SPECIFIC OBJECTIVES

The specific objectives of this thesis are:

1. To assess the state of knowledge on hyperparasitic fungi on Meliolales.

By the elaboration of a species checklist of hyperparasitic fungi on Meliolales around the world, the following questions can be addressed:

    a. How many species of hyperparasitic fungi on Meliolales are known from literature?

    b. What are the ecoregions where hyperparasitic fungi on Meliolales have been studied the most?

    c. What are the patterns of distribution of known fungi?

    d. What are the knowledge gaps?

2. To comment on host-specificity patterns of hyperparasitic fungi, their fungal and plant hosts, through the development of tritrophic networks.

3. To contribute to the systematic and knowledge about the evolution of hyperparasitic fungi, by generating DNA sequences for the first time.

4. To describe new species and records of hyperparasitic fungi for Benin and Panama through an integrative approach that includes detailed morphological descriptions and illustrations, as well as molecular sequence data.

MATERIALS AND METHODS

Taxon sampling

Fresh samples of leaves infected with black mildews were opportunistically collected by the author and collaborators in Western Panama from January−March 2020, and in Benin, West Africa, in February and September−October 2022. The sampling areas in each country are shown as geographical points in Figure 4. Besides the newly collected specimens, 35 historical specimens, including 12 type species, were examined for the present work. These specimens were loaned from the following herbaria: F, FH, IMI and PRM
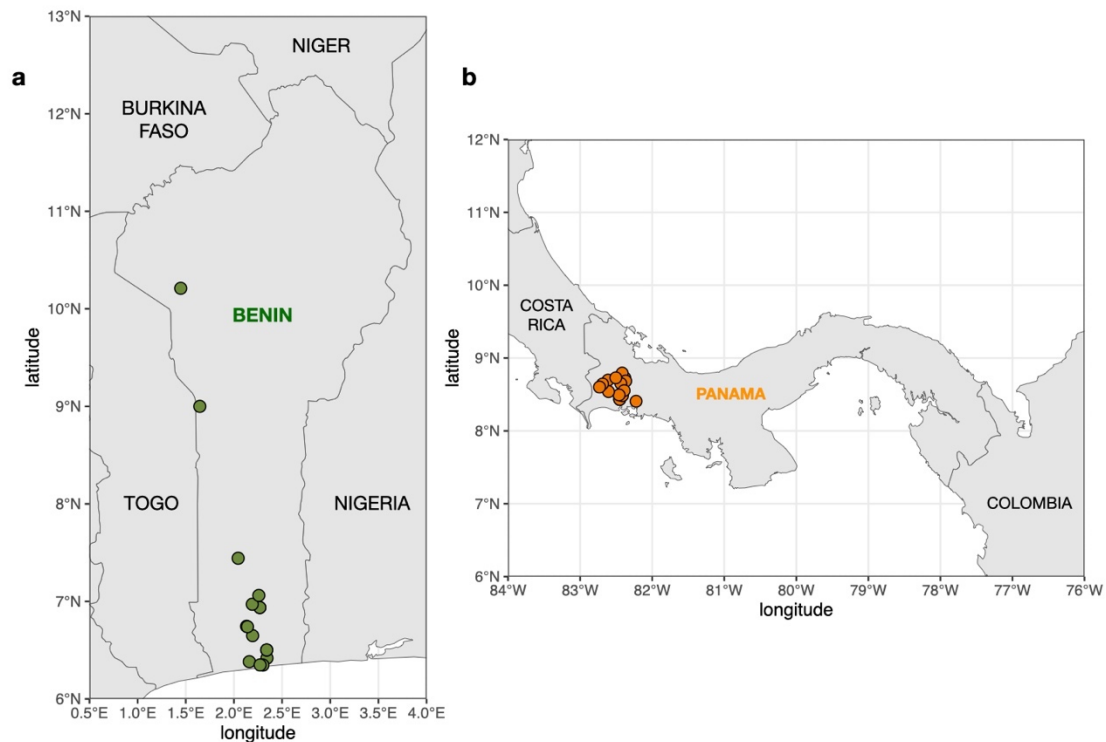
Figure 5 | **Collection sites included in the present study. a** Benin; **b** Panama. Each dot represents a sampling event (n = 32, 16 per country).

Infected leaves were dried in a plant press and deposited in the herbarium at the Universidad Autónoma de Chiriquí (UCH, specimens from Panama) or in the mycological herbarium of the University of Parakou (UNIPAR) in Benin. If a given sample was large enough, a duplicate was deposited in the Botanische Staatssammlung München (M).

Microscopic analyses

Dried specimens were observed by stereomicroscopy and by light microscopy (LM). Measurements of at least 20 conidia or ascospores have been made for each specimen at magnifications of ×600 and ×1000. Measurements are presented as mean value ± standard deviation with extreme values in parentheses. Line drawings were made freehand on scaled paper. Images and drawings were edited with Photoshop (Adobe, San Jose, California). Specimens were also analysed morphologically by scanning electron microscopy (SEM). Materials used for SEM were prepared according to Hofmann et al. (2010). Illustrations included in this document and in the publications were drawn by the author and edited with Photoshop (Adobe, San Jose, California) and Illustrator (Adobe, San Jose, California).

Host plant identification

Host plants were identified by morphological characteristics and, in some cases, by molecular methods. Morphological identifications were made by comparison with herbarium specimens, literature (e.g., Akoègninou et al. 2006, Condit et al. 2011) and with the help of local botanists. More details on the DNA extraction and PCR methods for identification of host plants are given in **Bermúdez-Cova et al. (2023b)** and in **Manuscript 1**.

Cultivation of hyperparasitic fungi

More than 50 cultivation attempts of hyperparasitic fungi were done in artificial media in Benin, Panama and in Frankfurt am Main, Germany. The culture media tested and their components are shown in Table I.

Table I. Recipes for cultivation media tested in the present study for the cultivation of hyperparasitic fungi.

Medium	Nutrients	Antibiotics	Agar	Reference
Malt extract agar (MEA) 2 %	Malt extract 20g	Tetracycline 0.5 g or Chloramphenicol 0.4 g	15 g	Modified from Wu et al. (2000)
Potato dextrose agar (PDA)	Potato extract (solid) 4 g Glucose 20 g	Chloramphenicol 25 mg	15 g	Gams et al. (1988)
V8-PDA agar	V8 juice 150 ml PDA 10 g CaCO$_3$ 3 g Yeast extract 4 g	Chloramphenicol 25 mg	10 g	Lamari and Bernier (1989)

DNA extraction, amplification and sequencing

DNA of hyperparasitic fungi was isolated directly from the synnemata, apothecia, perithecia or pseudothecia of fresh, recently collected samples from Benin and Panama. Numerous attempts (> 100) were made to obtain DNA from all the specimens included in this study. Except for the species cited in **Bermúdez-Cova et al. (2023b)** and **Manuscript 1**, these attempts were unsuccessful. No attempts to extract DNA from herbarium specimens were carried out because the historic specimens had no (or a few) fungal tissue, the colonies were too old/contaminated, or no permissions from herbaria were granted.

As no single suitable protocol for the extraction of hyperparasitic fungi exists, I tested some methods, parameters and kits for the extraction, amplification and sequencing of the samples collected in this study. A summary of these aspects is shown in table II.

Table II. List of methods, kits and parameters tested for the DNA extraction, amplification and sequencing of the samples collected in this study.

Methods/Kit	Phase	Reference or supplier
Liquid nitrogen.	Pre-treatments	van Burik et al. (1998), Haugland et al. (1999)
Mechanical disruption.	Pre-treatments	Meswaet et al. (2021)
Enzymatic digestion.	Pre-treatments	Glee et al. (1987)
E.Z.N.A. forensic kit.	DNA extraction	Omega Biot-tek
Phenol-Chloroform	DNA extraction	Kumar and Mugunthan (2018)
Increased concentration of BSA.	PCR	Eckhart et al. (2000)
REPLI-g Single Cell kit	PCR	Qiagen

The target regions for amplification were the nrSSU, nrITS and nrLSU rDNA. The primers and protocols used for PCR reactions, as well as for sequencing, are described in **Bermúdez-Cova et al. 2023b** and in **Manuscript 1.**

Phylogenetic analyses

Consensus sequences of trace files were generated with Geneious 10.2.2 (https://www.geneious.com, Kearse et al. 2012) and searched against GenBank (https://www.ncbi.nlm.nih.gov/, Benson et al. 2014) with MegaBLAST. Ambiguous and miscalled bases were corrected, when possible, after examination of the corresponding chromatogram files. Sequences with a high similarity were aligned with MAFFT v. 7 using the L-INS-I algorithm (Nakamura et al. 2018). The alignments were manually checked by using MEGA v. 7 (Kumar et al. 2016). Gblocks v. 0.91b (Talavera and Castresana 2007) was used to remove poorly aligned positions and divergent regions from the DNA alignment. Phylogenetic analyses of this study were conducted by applying maximum likelihood (ML) in RaxML-HPC2 v.8.2.12 (Stamatakis 2014) on XSEDE (Miller et al. 2010) and Bayesian phylogenetic inference with the program MrBayes 3.2.6. (Ronquist et al. 2012) on XSEDE (Miller et al. 2010), available on the CIPRES Science Gateway web portal (http://www.phylo.org/sub_sections/portal/).

Tritrophic networks

To illustrate the interactions between the known species of hyperparasitic fungi on black mildews, their meliolalean hosts and their host plants, I performed a network analysis. The network was visualized using the packages ggforce v0.3.3 (Pedersen 2021) and ggplot2 v3.3.5 (Wickham 2016) in R v4.1.2 (R Core Team 2022). For more details on this tritrophic network analysis, see **Bermúdez-Cova et al. 2022.**

Species accumulation curves, species richness and maps

The packages maps v3.4.0 (Becker et al. 2021), rnaturalearth v0.1.0 (http://github.com/ropen scilabs/rnaturalearth) and ggplot2 v.3.3.5 (https://ggplot2.tidyverse.org) were used to draw maps of the different ecoregions and study areas. Functions in the package vegan v2.5-7 (Oksanen et al. 2020) were used to build curves of species accumulation with sampling covering, based on the number of records (see **Bermúdez-Cova et al. 2022**) and species (Fig. 7). Total expected species richness was assessed using Chao (Chao 1984), Jack1 (First order jackknife), Jack2 (Second order jackknife) and bootstrap estimators (Smith and Van Belle 1984, Chao 1987, Palmer 1990, Colwell and Coddington 1994, Walther and Morand 1998).

MAIN RESULTS AND DISCUSSION

An overview of the specimens and species collected is provided to show the high diversity of hyperparasitic fungi on Meliolales found in the context of the present investigation. Moreover, the major findings of the author's publications and manuscripts are placed into a wider context that will include the most important results regarding the morphology, systematics, ecology and evolution of hyperparasitic fungi that grow on colonies of Meliolales.

STATE OF KNOWLEDGE: SPECIES CHECKLIST

In **Bermúdez-Cova et al. 2022**, I compiled the information of 525 records distributed in 86 publications, to elaborate a species checklist of species of fungi known to be hyperparasitic on Meliolales. Approximately 189 species of hyperparasites are known, but the number of existing species is expected to be higher. The species checklist showed some relevant aspects about the study of these fungi. These aspects constituted the basis of this research:

a. Information on hyperparasites of black mildews is scattered through literature, and the study of these fungi abruptly stopped after 1980. As a result, hyperparasitic fungi were only described by morphology, before the widespread use of molecular techniques in fungal taxonomy. In addition, the little attention that has been given to systems involving hyperparasites of Meliolales during the last 20 years could be explained by the fact that black mildews are not aggressive parasites, and that they mainly infect ornamental plants (Sandhu et al. 2021). Attention is currently focused on the study of hyperparasites capable of reducing infections caused by crop pathogens, such as rust fungi (Rosenheim et al. 1995, Day 2002).

b. Hyperparasitic fungi of Meliolales belong to different systematic groups. However, the systematic position of a large number of species is unknown. 110 species are "incertae sedis" ("uncertain position") for one or several levels of classification. The systematics of hyperparasitic fungi has been proposed solely on the basis of morphological characters, and DNA reference sequences of hyperparasitic fungi on Meliolales are lacking in public databases. More details on the challenges involved in the molecular study of these fungi can be found in **Bermúdez-Cova et al. (2022, 2023a)** and in the upcoming sections.

c. Species of hyperparasitic fungi on Meliolales appear to be distributed in the Neotropics, Afrotropics and Indomalayan ecoregions, suggesting a pantropical distribution by these organisms.

d. There is a dearth of data regarding the known distribution of hyperparasitic fungi per country in the tropics and, as mentioned before, there were no species known for Benin and Panama before the present investigation.

Checklists on species diversity are important sources of information for the characterization of biodiversity in a given area. They help to understand the present state of knowledge of fungi in an area, to decide whether a record of a fungal species is new for the area, to provide numbers for the comparison of biodiversity among regions and countries (Piepenbring et al. 2020, Ferraro et al. 2022). In addition, species checklists are particularly important to identify undersampled taxonomic or ecological groups, such as hyperparasitic fungi on Meliolales, as well as poorly explored geographical areas. Although they represent a very valuable tool, little scientific value is given to them in the mycological community.

DIVERSITY OF HYPERPARASITIC FUNGI ON MELIOLALES IN BENIN AND PANAMA

A total of 86 samples of plant specimens with black mildew infections were collected by the author. From this number, 44 specimens were parasitized by hyperparasitic fungi. 14 specimens collected by A. Sanjur, A. Tabé, A. Krauß and M. Piepenbring were also included in this study. The 58 specimens comprise approximately 31 species of hyperparasitic fungi. In total, 55 % of the collection (17 species) have been identified at the species level and 16 % of the collection (5 species) at the genus level, whereas 29 % of the collection (9 species) remain as morphospecies. The morphospecies could not be identified due to the presence of sterile hyphae only. Nine species have been newly recorded for Benin, seven for Panama, one for Africa and two for mainland America. Furthermore, four species are new to science: one species, *Paranectria longiappendiculata*, is described by **Bermúdez-Cova et al. (2023b)**, and three species of the genus *Spiropes* are described in **Manuscript 1**. Examples of the diversity of hyperparasitic fungi collected in Benin and Panama are shown in figure 6.
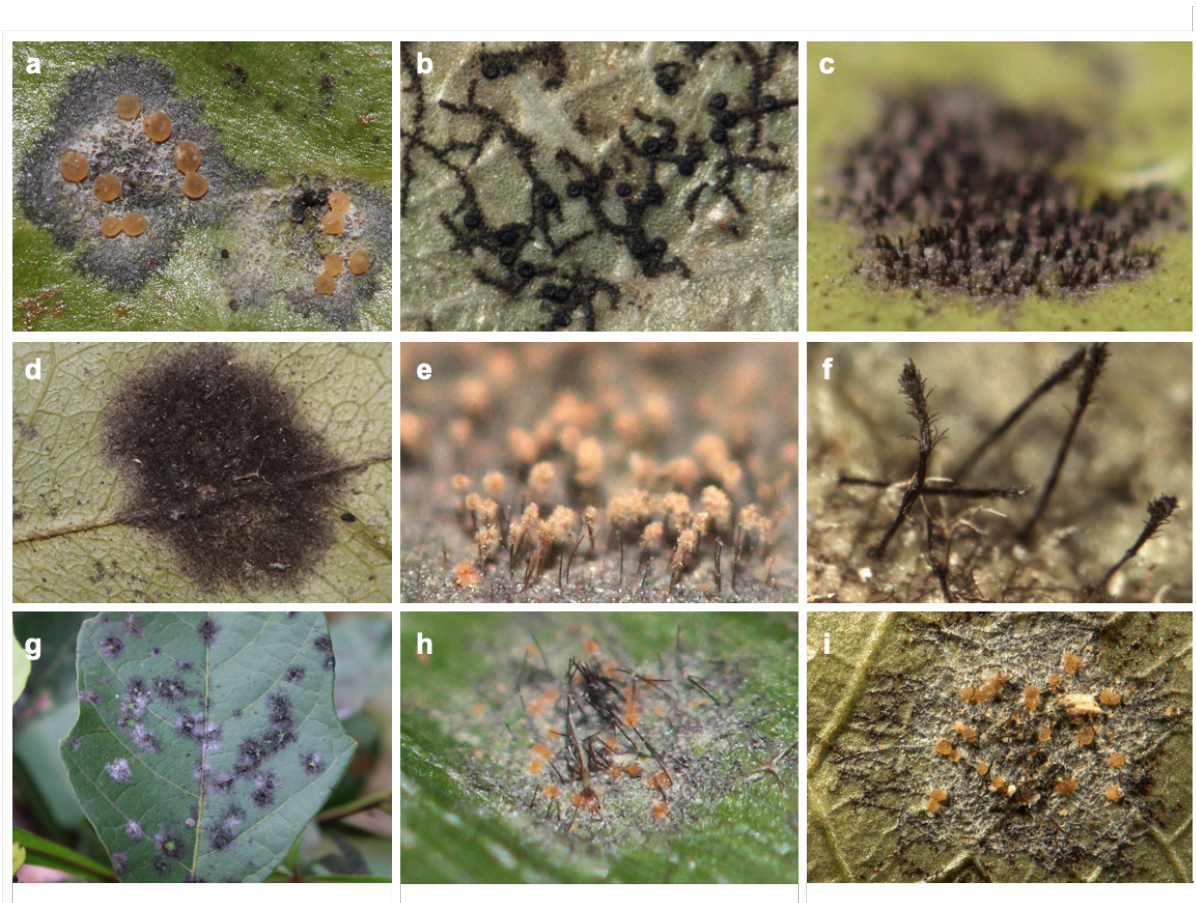
Figure 6 | **Some examples of the diversity of hyperparasitic fungi growing on colonies of Meliolales in Benin and Panama. a** Apothecia of *Calloriopsis herpotricha*; **b** Catathecia of *Trichothyrium* sp.; **c** Non-synnematous conidiophores of *Spiropes capensis* growing in groups; **d** Single conidiophores of *Spiropes carpolobiae* intermingled with the hyphae of a meliolalean host; **e** Synnemata of *Atractilina parasitica*; **f** Synnemata of *Spiropes japonicus*; **g** White hyphae of an *Acremonium*-like moniliaceous hyphomycete; **h** Perithecia of *Dimerosporiella cephalosporii*; **I** Pseudothecia of *Paranectriella* sp.

For 32 sampling events, 16 events per country, the species accumulation curves do not reach saturation for Benin and Panama (Fig. 7), suggesting that more species remain to be discovered in these countries. Moreover, species richness estimators (Chao, Jackknife 1, Jackknife 2 and Bootstrap) predicted 17–37 and 27–56 species of hyperparasitic fungi on Meliolales for Benin and Panama, respectively. The estimations varied strongly depending on the estimator function, and none of the estimators' curves approached saturation (Appendix II). The species richness was higher in Panama (22 species) than Benin (13 species). The most common species in Panama was *Spiropes melanoplaca*, whereas *Atractilina parasitica* was the most common species in Benin. Both species were repeatedly collected at different collection sites and dates. The perithecioid hyperparasite, *Dimerosporiella cephalosporii*, was the most common species in both countries. Other species, e.g., *Paranectriella* sp., however, were represented only by few or a single specimen.
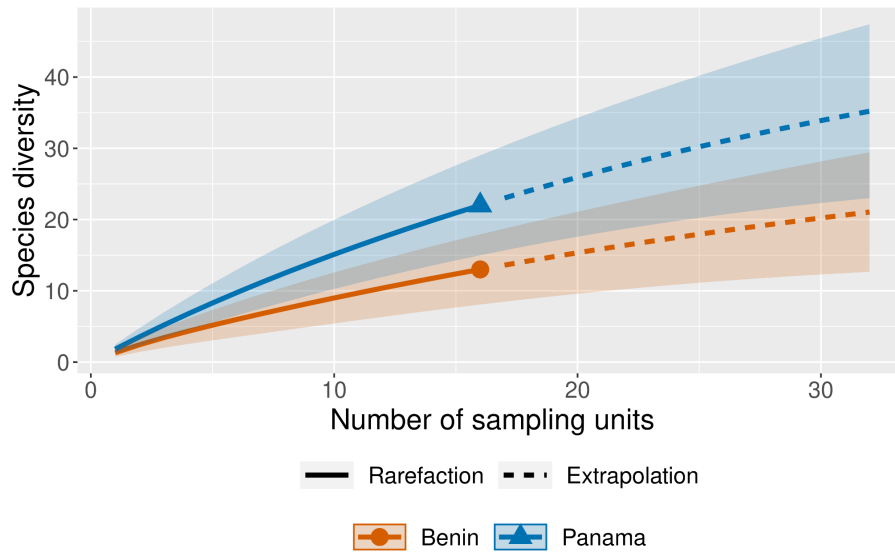
Figure 7 | Sample-size-based rarefaction (solid lines) and extrapolation (dotted lines) curves as functions of number of species of hyperparasitic fungi per sampling event in Benin and Panama. The curves are based on Hills numbers with q = 0. The solid triangle and dot represent the reference samples, and the shaded area corresponds to the 95 % confidence interval.

Hyperparasitic fungi distribute alongside their hosts (Sun et al. 2019). As the species diversity of Meliolales is higher in the tropics than in extratropical latitudes (e.g., Piepenbring et al. 2011), and hyperparasitic fungi appear to be restricted to meliolalean hosts, we expect to find a high diversity of hyperparasites in the tropics as well. In fact, the findings presented in this thesis show a surprisingly high diversity of hyperparasites in Benin and Panama, based on only a few months of fieldwork. Further sampling activities will certainly increase our knowledge on hyperparasitic fungi on Meliolales.

SYSTEMATICS OF HYPERPARASITIC FUNGI ON MELIOLALES

Hyperparasitic fungi on Meliolales do not represent a single systematic group, but rather an ecological group of fungi. This is evident from the wide variety of reproductive structures that these fungi produce, such as apothecia, catathecia, perithecia, pseudothecia, pycnidia, synnemata, among others. A brief description of the morphological groups of hyperparasitic fungi and their most common species is given in **Bermúdez-Cova et al. 2022**. All hyperparasitic fungi on Meliolales are species of Ascomycota (Bermúdez-Cova et al. 2022). They mainly belong to the Dothideomycetes and Sordariomycetes, with a few species in the Leotiomycetes and, after this work, one species in the Lecanoromycetes, namely *Calloriopsis herpotricha* (see **Bermúdez-Cova et al. 2023b**). As mentioned above, more than 50 % of the

species of fungi known to be hyperparasitic on Meliolales remain as incertae sedis. For the present work, I focused on the systematics of perithecioid hyperparasites, and the dematiaceous hyphomycetes of the genera *Atractilina* and *Spiropes*, as they were the most common groups of hyperparasitic fungi on Meliolales in Benin and Panama.

Morphological characters have been used over the last 300 years to identify, classify and infer the relationships of fungi (Hyde et al. 2010). They are used for classification of fungi at the level of order or family, but may not work well for species classifications (Lutzoni et al. 2004, Raja et al. 2017). However, in cases where there is a lack of DNA sequence data, such as in the case of hyperparasites of Meliolales, morphological data may still be useful to infer systematic relationships (Chethana et al. 2020). In **Bermúdez-Cova et al. 2023b**, the systematic position of five perithecioid hyperparasites was discussed, based on morphological characteristics and, for some species, confirmed by DNA molecular data. For the newly proposed species of perithecioid hyperparasite, namely *Paranectria longiappendiculata,* for example, it was not possible to observe the asci in any of the specimens collected. Therefore, it was not possible to assign the species to either the Dothideomycetes or the Sordariomycetes, as fungi of both taxa mainly differ by producing bitunicate and unitunicate asci, respectively (Lutzoni et al. 2004). However, other morphological features, such as the cells of the ascomatal walls, resembled those of species of hypocrealean fungi within the Bionectriaceae and Nectriaceae (Rossman et al. 1999). This hypothesis was later confirmed by DNA sequence data.

Currently, it is common to combine morphological and molecular data to resolve taxonomic problems and provide a more holistic approach towards a classification system that reflects past evolutionary pathways, as morphology-based identification may lead to incorrect systematic relationships (Jayasiri et al. 2015). For example, the apothecioid fungus *Calloriopsis herpotricha* (see **Bermúdez-Cova et al. 2023b**) was previously classified in the Phacidiales, Leotiomycetes, based on the morphological characteristics of the apothecium (Pfister 1976, Baral and Marson 2001). However, the ITS and SSU DNA sequences generated for the first time for this species in the context of the present study revealed that *C. herpotricha* is most probably a member of the Ostropales in the Lecanoromycetes. Some lineages of non-lichenized Ascomycota are known to be derived from lichenized ancestors by the loss of the lichen symbiosis in favor of a saprotrophic, lichenicolous, or parasitic mode of nutrition (Lutzoni et al. 2001, Hawksworth 2015, Honegger 2022). Examples of these include non-

lichenized members of Arthoniales (Arthoniomycetes) and Ostropales (Lecanoromycetes; Kendrick 2017). The foregoing and the fact that the sequences I obtained clustered together with other sequences of members of the Lecanoromycetes confirms the placement of *C. herpotricha* in this class and not in the Leotiomycetes. This represents the first report of a hyperparasitic fungus in this class (**Bermúdez-Cova et al. 2023a**).

The systematics of hyperparasitic fungi on Meliolales can also be inferred by using ecological data. In **Manuscript 1**, for example, the systematic position of *Atractilina parasitica*, a very common hyperparasite of Meliolales, is discussed not only based on morphology and molecular data, but also based on its possible anamorph-teleomorph connection. *Atractilina parasitica* is a synnematous hyphomycete that has been recorded only in black mildews colonies, especially in Africa (Deighton and Pirozynski 1972, **Bermúdez-Cova et al. 2022**). This fungus is characterized by the presence of golden-brown true synnemata, denticulate conidiogenous loci, pale pluriseptate conidia and a hyperparasitic lifestyle (Deighton and Pirozynski 1972). Before the present investigation, the systematic position of this species was unclear (Incertae sedis, Ascomycota). In the past, Hansford (1941, 1946) reported specimens of *A. parasitica* growing together with the perithecioid fungus *Malacaria meliolicola* (Dothideomycetes, Ascomycota). However, this anamorph-teleomorph connection was made only on the basis of the frequency of occurrence of both species in the same colonies of Meliolales. In Benin, both species were also found growing together on leaves of *Coffea arabica*. The LSU DNA sequences of *A. parasitica* obtained for the first time showed a 98 % percentage of similarity with the newly generated sequences of *M. meliolicola*. Therefore, the systematic position of *A. parasitica* in the Dothideomycetes and the anamorph-teleomorph connection between these two species are confirmed. *Atractilina parasitica* may also belong to the order Pleosporales s.l., but in order to confirm this systematic hypothesis and to determine the placement of *A. parasitica* at family level, the use of multiple loci is necessary in the future.

There are also cases where the systematics of certain species can only be resolved after a careful re-evaluation of the concepts of genera and species. As an example, in **Manuscript 1**, a systematic revision of the 19 species of the genus *Spiropes* Cif. Hyperparasitic on Meliolales was done. This genus currently comprises morphologically highly heterogenous species that are not congeneric with the type specimen. As a result, it is possible to find species with a wide range of conidiophores types, conidiogenesis and conidia to be included in *Spiropes*. The preliminary DNA sequence data generated in this publication suggests that the species of

Spiropes included in the analyses may belong to the Leotiomycetes. However, this result only includes 10 % of the species of the genus known to be hyperparasitic on Meliolales, and 6 % of the total number of species of the genus *Spiropes*. Instead of describing more and more species as part of the genus, a re-evaluation of the natural concepts of the genus is needed. *Spiropes* is currently a repository genus of highly heterogeneous species, and it may be split in the future, once the genera and species concepts of the genus are validated by morphology and molecular methods. This situation is similar for species of the genus *Atractilina* and, most probably, for many other groups of hyperparasitic fungi. A detailed discussion of this topic can be found in **Manuscript 1.**

The results obtained in the context of the present investigation represent the first contributions towards the study of the systematics of hyperparasitic fungi on Meliolales. A broader taxon sampling, a re-evaluation of existing genera and species concepts and the analysis of additional gene regions will be necessary to enhance the resolution of the study of these organisms in future studies. A phylogenetic hypothesis of the systematic groups where hyperparasitic fungi of Meliolales can be found is presented in Fig. 8. The new data generated in the context of the present investigation is highlighted in the tree.

CHALLENGES OF MOLECULAR STUDIES OF HYPERPARASITIC FUNGI ON MELIOLALES

DNA of 20 specimens of hyperparasitic fungi on Meliolales, representing seven species, has been isolated in the context of this investigation. In total, ten sequences were generated by the author (six for nrLSU, three for nrITS and one for nrSSU). More than 50 attempts were done in order to extract, amplify and sequence the DNA of all the specimens collected. Apart for the results mentioned above, these attempts failed. From all the methods tested in table I, the freezing method and the mechanical disruption were the pre-treatments that worked best. As for the DNA extraction method, the E.Z.N.A. forensic kit was the most sensitive and appropriate for small amounts of fungal material.
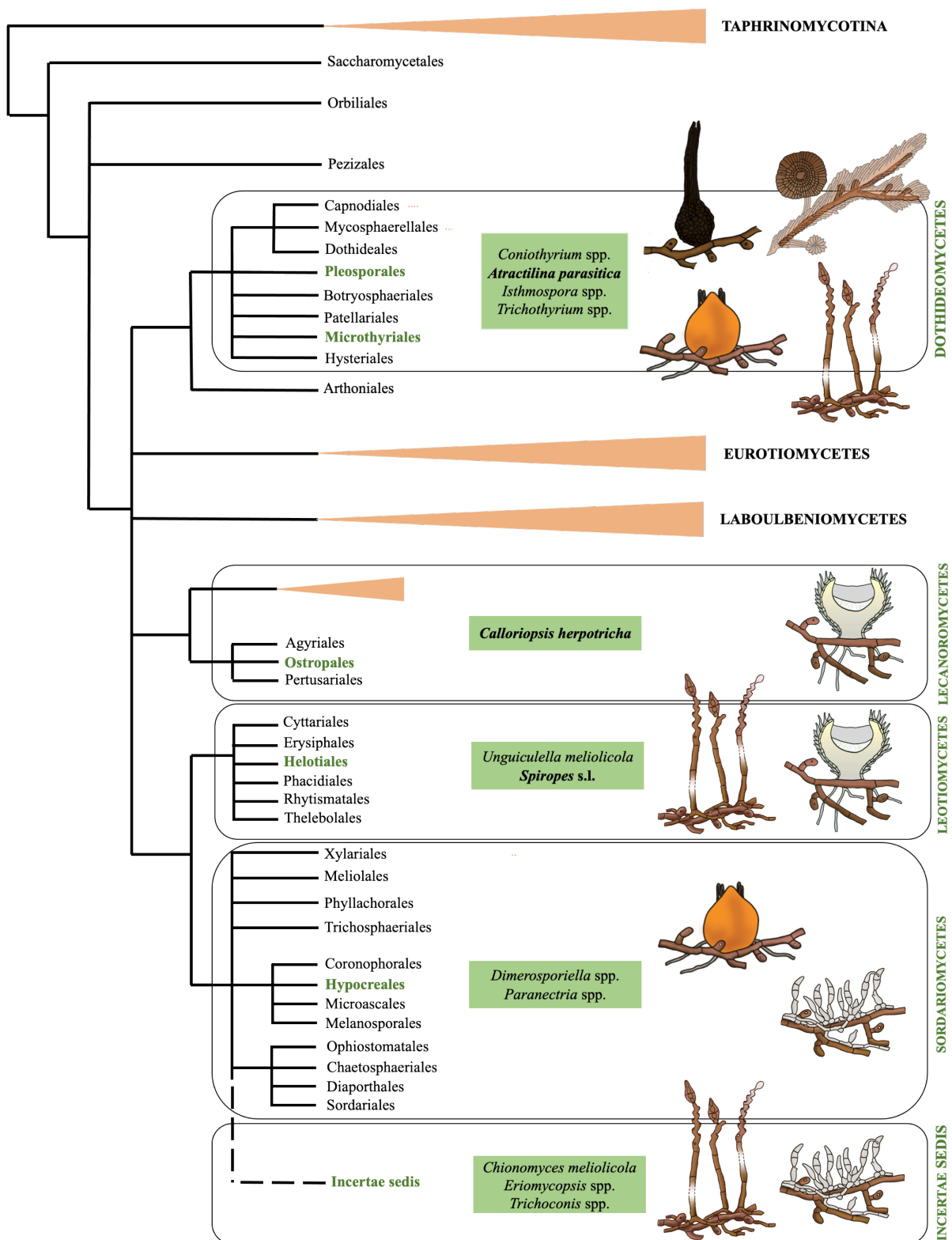
Figure 8 | Phylogenetic hypothesis of species of hyperparasitic fungi of Meliolales in the Ascomycota tree. Classes and orders written in green include species known to be hyperparasitic on black mildews. In each class, within green boxes, the most common species of hyperparasites are included. Species that represent new contributions generated in the context of this research are written in bold. Each class also includes an illustration referring to the predominant type of reproductive structures of the hyperparasites.

The molecular study of hyperparasitic fungi remains a challenging process. As they belong to different systematic lineages and have different morphologies, no specific set of molecular methods have been developed to study them (**Bermúdez-Cova et al. 2023a**). And the reality is that it is probably not possible to develop a single method for its study. In **Bermúdez-Cova et al. (2022, 2023a)** an extensive discussion of the problems that scientists encounter when studying hyperparasitic fungi was made. The two most relevant challenges are discussed below:

a. As hyperparasitic fungi are part of multitrophic interactions (see below the subsection *Multitrophic interactions*), it is common to find the hyperparasites intermingled with tissue and/or cells of the meliolalean host and other organisms present in the same colony (**Bermúdez-Cova et al. 2022, 2023a**). This makes the isolation of DNA exclusively from the hyperparasite difficult.

b. Public databases are notoriously lacking DNA reference sequences of hyperparasitic fungi on Meliolales (**Bermúdez-Cova et al. 2022, 2023a**). As a result, the DNA sequences obtained in the context of the present investigation could be related to existing species concepts only based on morphology. It is necessary to increase the efforts to recollect specimens of hyperparasitic fungi and to generate more DNA sequences in the future.

ECOLOGY OF HYPERPARASITIC FUNGI ON MELIOLALES

Host specificity

Hyperparasitic fungi on Meliolales are most probably biotrophic parasites, as they have been found in the field only growing together with the fungal host, and there are no records of cultivation on artificial media (**Bermúdez-Cova et al. 2022**). All the attempts to cultivate the specimens from Benin and Panama on artificial media failed. The morphological analyses by LM and SEM of the specimens collected clearly showed that these fungi establish an intimate contact with the hyphae (Fig. 8) and the spores (**Bermúdez-Cova et al. 2022**) of the meliolalean hosts without the presence of haustoria.
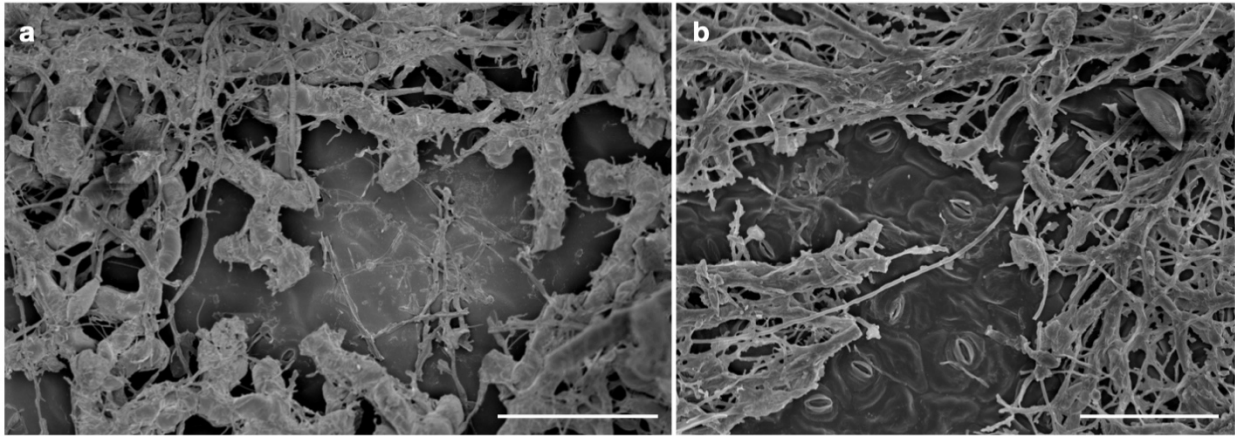
Figure 9 | Interaction between the hyphae of hyperparasitic fungi and the hyphae of their meliolalean hosts, as seen by SEM. a Hyphae of *Spiropes clavatus* growing on the hyphae of *Meliola* sp.; **b** Hyphae of *Spiropes dorycarpus* growing on the hyphae of *Meliola* sp. Notice that, in both cases, the interaction is restricted to both organisms and it does not involve the tissue of the plant hosts.

Network theory is another tool frequently used in ecological research to understand the specificity of species interactions. In these networks, species are represented as nodes that form links or interactions (Pocock et al. 2016). Tritrophic parasitic networks have been conducted mostly on phytophagous insects and their insect parasites or parasitoids that infect them (Derocles et al. 2018, de Araujo and Maia 2021, Kawatsu et al. 2021). As for fungal systems, de Groot et al. (2020) analyzed the ecological interactions between bats, bat flies and hyperparasitic microfungi. Network theory, however, was not yet applied to fungal hyperparasitic-host fungus interactions before the present study. In **Bermúdez-Cova et al. (2022)**, the interactions of known genera of hyperparasitic fungi, their meliolalean hosts and their plant hosts were illustrated in a network. This network showed that species of hyperparasitic fungi are generalists concerning genera of Meliolales, i.e., the host range of most genera of hyperparasitic fungi includes several species of one or several genera of black mildews. Hyperparasitic fungi may also be specialists at the level of order, as many species have been found growing only on meliolalean hosts. However, the latter should be taken with caution, due to the existing dearth of data and undersampling of hyperparasites of Meliolales. Some species, for example, are represented in the network by a single record, leading to the erroneous conclusion that the species may be highly specific. Other species, such as *Atractilina parasitica*, have been recorded more than 60 times growing on different genera and species of Meliolales, and only once in *Balladyna* sp. (Balladynaceae, Ascomycota; Deighton and Pirozynski 1972). The specimen on *Balladyna* is most probably a different species and will be

placed into a different taxon once the genus *Atractilina* is re-evaluated. This issue on the re-evaluation of the genus *Atractilina* is discussed in **Manuscript 1.**

Building multitrophic ecological networks is a difficult task, especially in poorly studied and highly diverse systems (Derocles et al. 2018), as is the case for hyperparasitic fungi and black mildews. Sampling efforts need to be increased and data from more countries and host fungi should be included to strengthen future analyses of these species' interactions (Cazabonne et al. 2022). Moreover, existing concepts of genera and species need to be revaluated as well (see **Manuscript 1**).

Multitrophic interactions

According to Kiss (2001), an interaction involving a hyperparasitic fungus consists of three trophic levels: the plant host, the fungal host and the hyperparasite. However, in reality, these relationships involve complex multitrophic interactions. In the case of Meliolales, their colonies constitute a perfect ecological niche to be colonized and used by other organisms (Piepenbring et al. 2011, Piepenbring 2015). The surface of the setae and other cells of the black mildews is hydrophilic, resulting in a prolonged state of moisture of the colonies that allow the growth of a wide range of organisms (Fig. 9). During the course of this study, I observed tardigrades, mites, yeasts, algae, cyanobacteria and spores from other fungi.
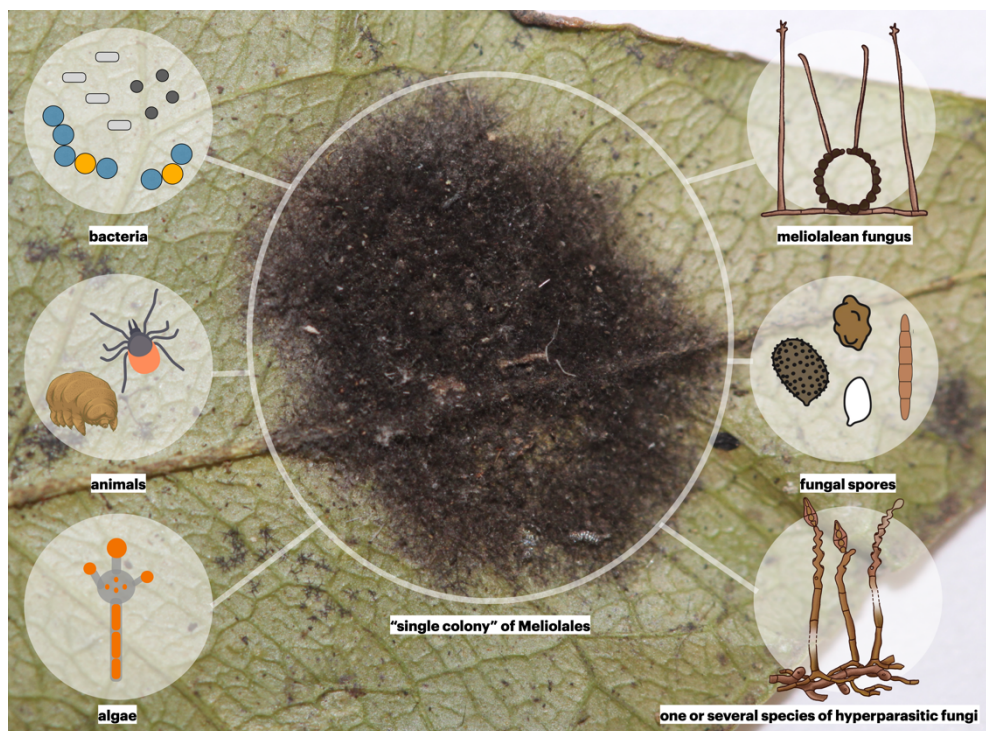
Figure 10 | Multitrophic interaction between the different cellular structures and organisms that can be present in a single meliolalean colony.

This multi-trophic relationship described above may also include the presence of more than one species of hyperparasites. Several species of hyperparasitic fungi can be found on the same leaf and even on the same colony of Meliolales (Stevens 1918, Ciferri 1955, **Bermúdez-Cova et al. 2023b**). For example, during the fieldtrip in Benin, it was possible to observe on leaves of *Coffea arabica* at least six different species of hyperparasites growing on the same leaves and even on the same colonies of Meliolales (Fig. 11).
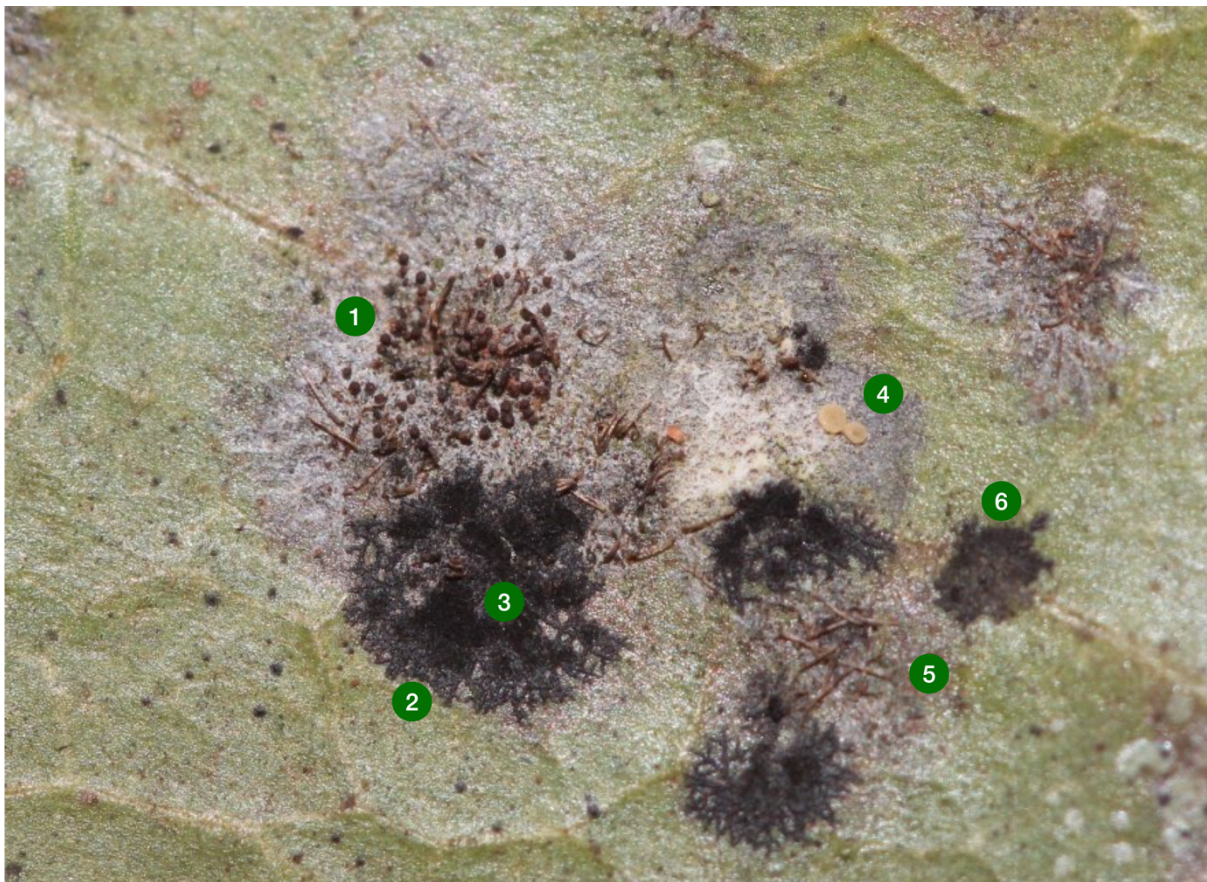
Figure 11 | Diversity of hyperparasitic fungi growing on a leaf of *Coffea arabica* and on the same meliolalean host. 1 Pseudothecia of *Malacaria meliolicola*; **2** Mycelial mat of *Trichothyrium* sp.; **3** Colonies of isthmospores of *Isthmospora trichophila*; **4** Apothecia of *Calloriopsis herpotricha*; **5** Synnemata of *Atractilina parasitica*; **6** Hyphae of *Spiropes* sp.

Patterns of distribution

Patterns of distribution of hyperparasitic fungi have been studied mainly for hyperparasites of rusts and powdery mildews (Zewdie et al. 2021), but never for those infecting species of Meliolales. The distribution of hyperparasitic fungi is restricted to that of their host (Sun et al.

2019). As Meliolales are restricted to tropical and subtropical areas (Piepenbring 2015), hyperparasites are expected to be found in these regions as well. It is also expected wide distribution areas of hyperparasitic fungi on Meliolales because of their broad spectra of host species (**Bermúdez-Cova et al. 2022**). In fact, the data collected in this thesis suggest that at least part of the species of hyperparasitic fungi of Meliolales have a pantropical distribution, as they have been recorded both in paleotropical and neotropical regions. This is consistent with the assumptions made by Samuels et al. (2002) regarding the pantropical distribution of tropical perithecioid fungi. Extensive additional fieldwork is needed in order to unravel distribution patterns of hyperparasitic fungi on meliolalean hosts.

OPEN ENDS: OTHER INTERESTING AND/OR POTENTIAL NEW SPECIES OF HYPERPARASITIC FUNGI ON MELIOLALES

There are some specimens of hyperparasitic fungi that could not be included in the main publications, mainly due to lack of time and material. It was not possible to identify them to the species level, and further morphological and molecular analyses are needed. These specimens are included here:

Potential new species

Dimerosporiella **sp.** (Fig. 12)

Specimen examined – On *Meliola* sp., on leaves of *Alchornea cordifolia* (Euphorbiaceae), Benin, West Africa, Lokoli, border of the forest, 7° 03' 41.6'' N, 2° 15' 24.9'' E, 17 m a.s.l., 21 Sep. 2022, A. Krauß, A. Tabé, O. Koukol, AK 27.

Colonies white, cottony, growing on *Meliola* spp. Hyphae septate, 1–2 μm wide, hyaline. Perithecia superficial, globose, (70–)108–120(–250) μm diam., yellow to orange, slightly translucent, not changing color in KOH, smooth; perithecial hairs arising from perithecial apex, non-septate, unbranched, 21–41 × 3–5 μm, wall 0.5–1 μm thick. Perithecial wall 8–11 μm wide, composed of small cells; perithecial apex formed by hyphae that grow outwardly to form perithecial hairs, and inwardly to form periphyses. Asci clavate, apex simple, (24–)32–35 × 5– 8 μm, 8–spored. Ascospores completely filling each ascus, biseriate, ellipsoidal to fusiform, sometimes guttulate, (8–)10–11(–12) × 2–4 μm, 1–3–septate, hyaline, smooth.

Notes – The specimen AK 27 differ from other species of the genus *Dimerosporiella* by the presence of long perithecial hairs, a morphological feature that has not been reported for any species of the genus (Rossman 1999). The closest morphological species to *Dimerosporiella* sp. is *D. pipericola*, but the asci are shorter (18–25 × 5–7 μm), and no information regarding the perithecial hairs size is provided in the original description of the species (Hennings 1904). The type specimen of *D. pipericola* needs to be compared with the specimen AK 27 in order to compare them properly.
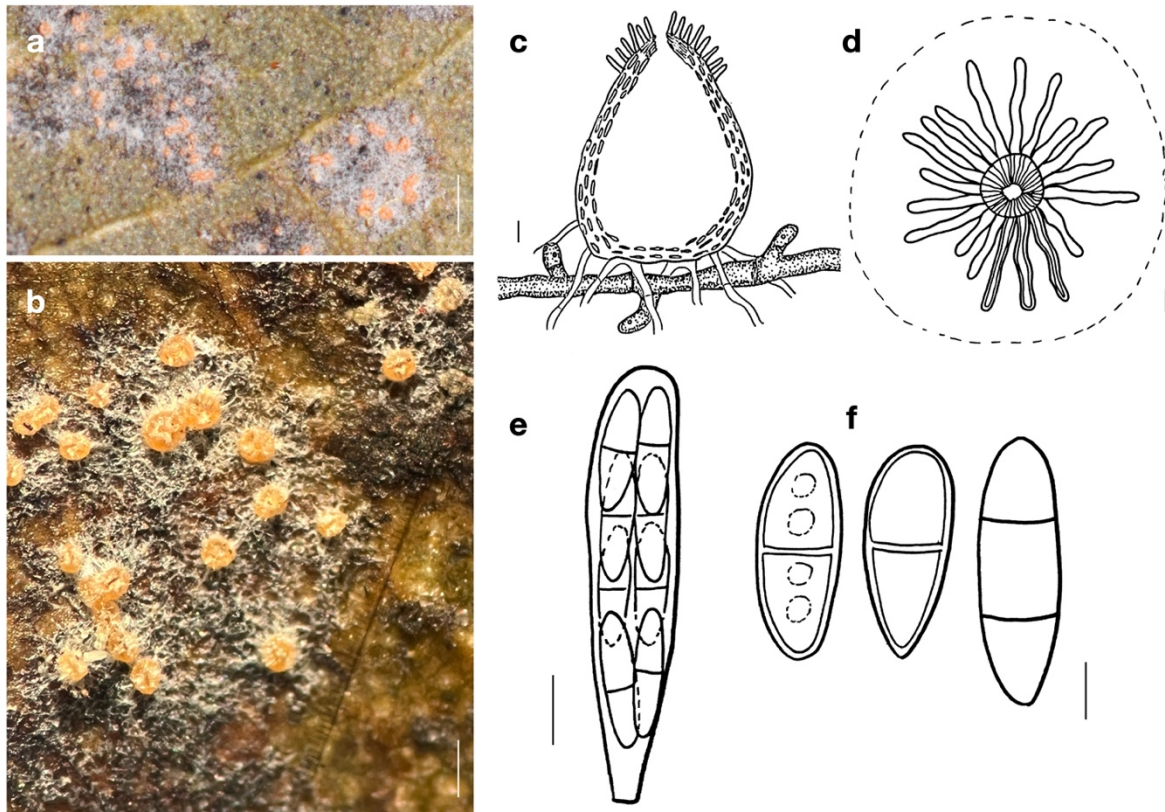

Figure 12 | *Dimerosporiella* **sp. (AK 27). a, b** Perithecia growing on colonies of *Meliola* sp.; **c** Perithecium on a hypha of *Meliola* sp.; **d** View from above of the ostiole surrounded by perithecial hairs. The thickness of the wall is only drawn in the three central bottom hairs; **e** Ascus with ascospores; **f** Ascospores. The thickness of the wall is drawn only on the first two spores. The guttulate content is only drawn on the left-hand side spore. Scale bars: 1 mm (**a**); 300 μm (**b**); 13 μm (**c**); 10 μm (**d**); 5 μm I; 2.5 μm (**f**).

Trichothyrium, a complex genus that needs morphological and molecular revision

The family Trichothyriaceae was introduced by Theissen (1914), and currently comprises four genera of fungicolous fungi, namely *Lichenopeltella* Höhn., *Macrographa* Etayo, *Pachythyrium* G. Arnaud ex Spooner & P.M. Kirk, and *Trichothyrium* Speg. (Hofmann 2010, Hongsanan 2020). Members of the family are characterized by the presence of catathecia, i.e.,

flattened perithecia with a well-developed upper and lower peridial wall, and densely packed hyphae that form bands covering hyphae of the host fungus. (**Bermúdez-Cova et al. 2022**).

The genus *Trichothyrium* comprises 17 species hyperparasitic on colonies of Asterinales, Meliolales and other foliicolous species of Ascomycota (Piepenbring 2015). The asexual morph of *Trichothyrium* are species of *Isthmospora*, although this anamorph-teleomorph connection has not been yet confirmed by molecular sequence data (Hyde et al. 2011). The delimitation of species in the genus is ambiguous, as only a few morphological characteristics are used, such as the size of ascospores (Wu et al. 2011, Hongsanan et al. 2020). DNA sequence data is not available for any of the species of this genus and the family Trichothyriaceae (Hongsanan et al. 2020).

During the sampling events in Panama, five specimens of *Trichothyrium* spp. were collected. However, it was possible to identify the specimens only as morphospecies, as there are no taxonomical keys that can summarize all the known species and their morphological features. By a preliminary revision of the species of *Trichothyrium* known from literature, I realized that there are too few morphological characters to delimit species, and a revision of the genus is necessary. This is a very common group of hyperparasitic on Meliolales, especially in the Neotropics, and attention should be given to these species in the future. An example of a species portrait of one of the specimens collected in Panama is provided below (Fig. 12).
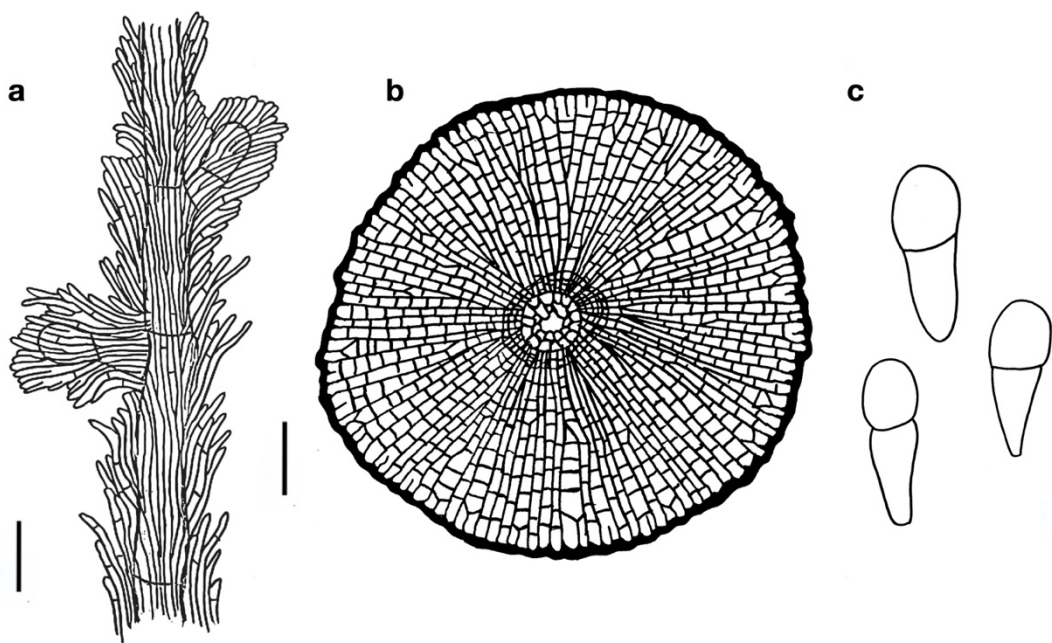

Figure 13 | *Trichothyrium* sp. (MB 93). a Hyphal mat; **b** Catathecium; **c** Ascospores. Scale bars: 5 μm (**a, c**); 10 μm (**b**).

IDENTIFICATION OF HYPERPARASITIC FUNGI ON MELIOLALES IN THE FIELD: IT'S ALL ABOUT THE PATTERNS

Hyperparasitic fungi can easily overgrow the entire colonies of the black mildews until the presence of the meliolalean host may be proved only by careful search under a light microscope (Stevens 1918). However, spotting these parasites in the field is not always an easy task. During the field trips, it was possible to identify the most common patterns for recognizing hyperparasites growing on colonies of black mildews. It is important to highlight the importance of knowing what a colony of Meliolales looks like (Fig. 1) before being able to recognize the presence of a hyperparasite. Colonies of black mildews are composed of superficial, dark, thin hyphae, with a distinctive black color (see white arrows in Fig. 11). Depending on the type of fungus parasitizing the meliolalean colonies, the recognition pattern will be different. Some examples frequently observed in the field were:

a. When colonized by **moniliaceous** or **perithecioid** hyperparasites (Fig. 14 a), the black colonies of the black mildews are covered by white hyphae, giving a grayish-white appearance to the colonies. Only by using a hand-lens it is possible to spot perithecioid ascomata

b. When colonized by species of *Spiropes* **or** *Spiropes*-**like** fungi (Fig. 14 b), the colonies of the black mildew appear as **dense colonies**, where the characteristic black color is replaced by a brown or purple-brown color.

c. When colonized by **synnematous species of** *Spiropes* (Fig. 14 c), the presence of the hyperparasite is easily recognized by the presence of hundreds of trichome-like structures. It is important to note that, when the hyperparasite is present, the fungal hyphae of the meliolalean host are covered by a brown mycelial mat, and it is not possible to recognize them without careful examination under the light microscope.

d. When colonized by *Atractilina parasitica* (Fig. 14 d), the black colonies of the meliolalean fungus are covered by golden-brown synnemata.

e. When colonized by species of *Trichothyrium* (Fig. 14 e), meliolalean colonies maintain their black color, but the hyphae appear to be thickened. The latter is due to the fact that

the hyphae of the Meliolales are covered by the mycelial mat of the catathecioid hyperparasite.
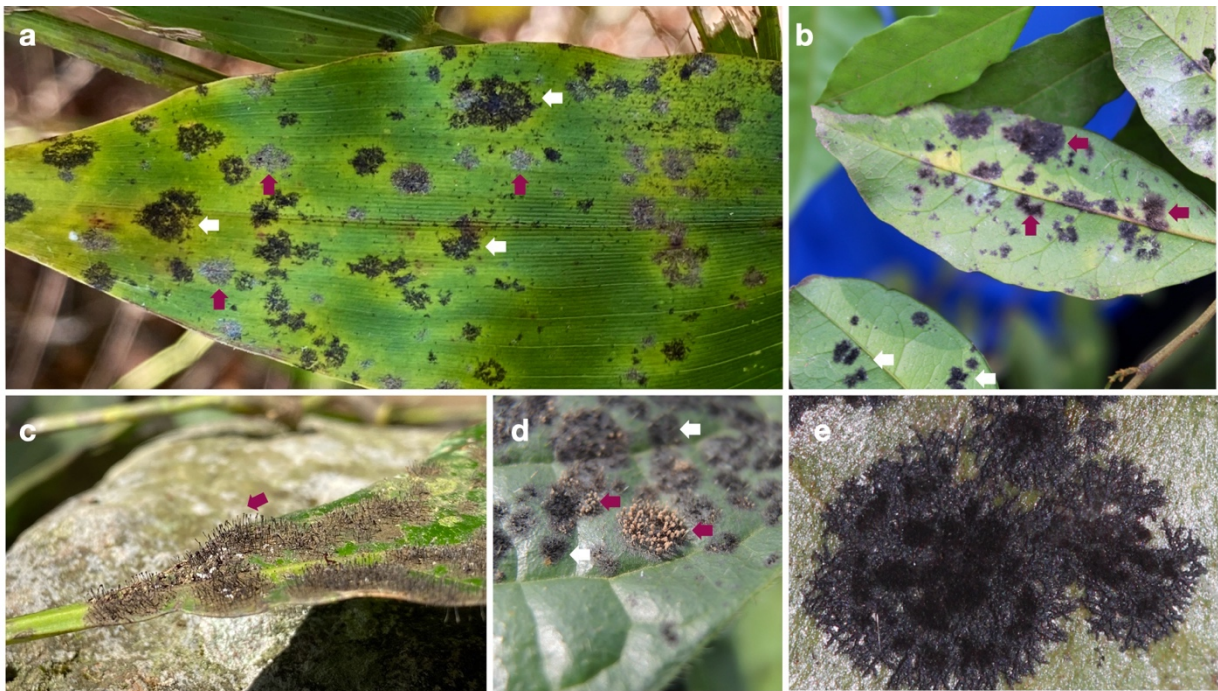

Figure 14 | Some of the most common patterns to identify hyperparasitic fungi on Meliolales in the field. A Surface of leaf of *Olyra latifolia* colonized by a meliolalean fungus. The black colonies are indicated by white arrows. Notice that some of the black colonies have a greyish appearance, as indicated by the fuchsia arrows, due to the presence of a hyperparasite. **B** Leaves of *Carpolobia lutea* infected by a meliolalean fungus. The black colonies are indicated by white arrows. Notice that some of the black colonies have a brownish or purple-brown appearance, as indicated by the fuchsia arrows, due to the presence of a hyperparasite. **C** A leaf of *Mangifera indica* colonized by *Meliola mangiferae*. The colonies are not easily distinguishable, but they are covered by the synnemata of *Spiropes melanoplaca*, giving the colonies a hairy appearance (see fuchsia arrow). **D** A leaf of *Clerodendrum capitatum* colonized by colonies of *Meliola clerodendricola.* The black colonies are indicated by white arrows. Notice that some of the black colonies have a golden appearance, as indicated by the fuchsia arrows, due to the presence of the synnemata of *Atractilina parasitica*. **E** A colony of *Meliola* sp. entirely covered by the mycelial mat of *Trichothyrium* sp. Notice the thickening of the hyphae.

Although each species of fungus has its own growth pattern, the patterns mentioned above remained constant in all specimens collected, and may be useful for a first recognition of hyperparasites of Meliolales in the field. As a personal consideration, the best way to identify a hyperparasite in the field is to make sure that the fungus is growing **ONLY** on the colonies of the parasite, and not on other surfaces (e.g., the surface of the leaves).

The following is a general taxonomic key to the most common genera of hyperparasitic fungi that grow on colonies of Meliolales in Benin and Panama. For a detailed key to species with

perithecioid ascomata, see **Bermúdez-Cova et al. 2023b**. For a detailed keys to species of *Atractilina* and *Spiropes*, see **Manuscript 1**.

Key to the most common genera of hyperparasitic fungi on Meliolales in Benin and Panama

1 Apothecia, catathecia, perithecia or pseudothecia present ….. 2
1* Ascomata absent ….. 7

2 Apothecia present …... *Calloriopsis* (see *C. herpotricha*)
2* Ascomata not apothecioid …... 3

3 Ascomata catathecioid. Superficial hyphae black, forming a mycelial mat that grows on the hyphae of the meliolalean host ….. *Trichothyrium*
3* Ascomata perithecioid 4

4 Perithecia with perithecial hairs around the apex. Asci unitunicate 5
4* Pseudothecia present. Asci bitunicate …... 6

5 Ascospores 1-septate, biguttulate. .... *Dimerosporiella*
5* Ascospores (up to) 3-septate, with appendages at their tips .... *Paranectria*

6 Ascospores mostly 3-septate, narrowly clavate, with an elongated base and rounded tips .... *Malacaria* (see *M. meliolicola*)
6* Ascospores (up to) 3-septate, with appendages at their tips .... *Paranectriella*

7 Hyaline, *Acremonium*-like conidia and conidiophores .... *Dimerosporiella*
7* Straw-coloured to dark conidia and conidiophores, synnematous and non-synnematous 8

8 Synnemata straw-coloured to pale olivaceous; conidiophores with denticulate conidiogenous loci; pale multiseptate conidia .... *Atractilina* (see *A. parasitica*)
8* Conidiophores synnematous and non-synnematous, dark brown to black, with cicatrized conidiogenous loci; conidia pigmented and multiseptate .... *Spiropes*

CONCLUSIONS

This dissertation represents advances in understanding the ecology, evolution, systematics and taxonomy of fungi hyperparasitic on colonies of Meliolales. The diversity of species and reproductive structures presented in this thesis are based on only three months of fieldwork and show a blatant lack of investigation on hyperparasitic fungi in the tropics. Taxonomic studies in this investigation generated four newly described species, seven new synonyms, nine new records for Benin and seven for Panama. The inventory of species in the context of the present investigation has increased the number of recent herbarium collections, species records and, for the first time, has provided molecular data of hyperparasitic fungi on Meliolales. These data will lay the groundwork for future studies focusing on hyperparasitic fungi on black mildews.

Almost all species of hyperparasites of black mildews were described in the past using only morphological characters. This resulted in the creation of different genera that are currently grouping species that are not necessarily congeneric with the type species of those genera. This thesis shows, using species of *Atractilina* and *Spiropes* as examples, the need for careful re-evaluation of the genera and species concepts, in order to better understand the systematics and evolution of hyperparasitic fungi.

The huge diversity of reproductive structures presented by hyperparasitic fungi on black mildews indicates the polyphyletic nature of this ecological group. As a result, no single method for molecular studies of hyperparasitic fungi can be developed and methods for extraction, amplification and sequencing of fungal DNA of hyperparasitic fungi will depend on multiple factors. These factors include the degree of contact between the hyperparasite and its fungal host, the type of reproductive structures, the degree of melanization of the cells, among others. The molecular study of these fungi continues to be a challenge. This thesis represents the first effort to develop a library of DNA sequence data of fungal hyperparasites of black mildews. Examples fund in this work emphasize that field work paired with molecular analysis still plays a crucial role for modern mycology, especially for challenging fungal groups, such as hyperparasites.

Extensive additional fieldwork is needed in order to better understand hyperparasitic fungi on Meliolales, and to unravel their distribution patterns, patterns of host-specificity, as well as new anamorph-teleomorph connections.

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Anlage 1

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Hyperparasitic Fungi on Black Mildews (Meliolales, Ascomycota): Hidden Fungal Diversity in the Tropics

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Hyperparasitism on plant-parasitic fungi is a widespread but rarely studied phenomenon. Here, for the first time, we compile in a checklist information provided by peer-reviewed literature for fungi growing on colonies of black mildews (Meliolales, Ascomycota), a species-rich group of tropical and subtropical plant-parasitic microfungi. The checklist contains information on 189 species of contact-biotrophic microfungi in 82 genera. They belong to seven morphological groups: dematiaceous hyphomycetes, moniliaceous hyphomycetes, pycnidioid, perithecioid, catathecioid, and apothecioid fungi. By the fact that species accumulation curves do not reach saturation for any tropical country, it is evident that the knowledge of the diversity of hyperparasitic fungi on Meliolales is incomplete. A network analysis of records of hyperparasitic fungi, their host fungi and host plants shows that genera of hyperparasitic fungi are generalists concerning genera of Meliolales. However, most species of hyperparasitic fungi are restricted to meliolalean hosts. In addition to hyperparasitic fungi, diverse further microorganisms use meliolalean colonies as ecological niche. Systematic positions of most species are unknown because DNA sequence data are lacking for species of fungi hyperparasitic on Meliolales. We discuss the specific challenges of obtaining DNA sequence data from hyperparasitic fungi. In order to better understand the diversity, evolution and biology of hyperparasitic fungi, it is necessary to increase sampling efforts and to undertake further morphological, molecular, and ecological studies.

Keywords: hyperparasitism, hyperparasitic fungi, Meliolales, checklist, Ascomycota, tritrophic interaction, network analysis, parasitism

1. INTRODUCTION

The term hyperparasite refers to an organism that parasitizes another parasitic organism. Hyperparasitism caused by fungi is rather widespread in nature, but it is a phenomenon that has been poorly studied (Haelewaters et al., 2018a, 2021). Several authors have reviewed this type of interaction (Barnett, 1963; Boosalis, 1964; Barnett and Binder, 1973; Cooke, 1977; Hawksworth, 1981; Haelewaters et al., 2018b; Sun et al., 2019). Fungi are able to parasitize parasitic organisms from different kingdoms (Moore et al., 2020). In this review, we consider fungi parasitic on plant-parasitic fungi. For a fungus to be considered a hyperparasite, it needs to impact the host fitness

through one or more modifications, otherwise it would be a hypermutualist or hypercommensal (Boosalis, 1964; Northrup et al., 2021).

Biotrophic plant-parasitic microfungi are frequently colonized by hyperparasitic fungi, many of which can penetrate the hyphae, the spores and/or the reproductive structures of their hosts (Gams et al., 2004). Some of these parasites attack specific groups of plant pathogens and are of interest as potential biocontrol agents, such as *Ampelomyces* spp., natural occurring hyperparasites of powdery mildews (Huth et al., 2021). The most common hosts include powdery mildews (Erysiphales), black mildews (Meliolales), rusts (Pucciniales), smuts (Ustilaginales), and Phyllachorales (Hawksworth, 1981; Gams et al., 2004). For the present review, we focus on hyperparasitic fungi on species of Meliolales.

Meliolales (Sordariomycetes, Ascomycota) form a large order of biotrophic, obligate parasitic fungi in the tropics and subtropics. It comprises 3,064 species, with *Meliola* being the most species-rich genus (1701 spp.; Jayawardena et al., 2020). Species of the order develop on leaves, petioles, twigs and sometimes fruits of vascular plants (Piepenbring et al., 2011; Hongsanan et al., 2015; Zeng et al., 2017). They are known as "black mildews", as they produce black colonies that are composed of dark, thick-walled, branched, superficial hyphae (**Figure 1**; Rodriguez Justavino et al., 2015). These hyphae carry numerous short, lateral branches called hyphopodia. Capitate hyphopodia are formed by a foot cell and a globose/lobate terminal cell. This terminal cell acts as an appressorium. A peg formed by the appressorium penetrates the leaf surface and forms a haustorium inside the epidermal host cell to absorb nutrients. Other lateral branches, the phialides, consist of a single, bottle-shaped cell, which can form small spores at the tips. These spores can function as conidia or spermatia, but they have been poorly studied. Meliolalean fungi form perithecia containing asci with dark brown, transversely septate ascospores. Most species present long setae attached to superficial hyphae and/or perithecia (Piepenbring et al., 2011; Piepenbring, 2015).

Infections by species of Meliolales result in a reduction of chlorophyll, starch, sugar, proteins and aminoacids in the affected areas of the plant host (Hosagoudar et al., 1997; Old et al., 2003; Rodriguez Justavino and Piepenbring, 2007). Respiration rates and the temperature of the infected areas may increase due to the lesions and the black color. Photosynthetic activity may be reduced (Hosagoudar et al., 1997; Hongsanan et al., 2014). Heavy infections caused by Meliolales result in a "dirty" appearance of the hosts, thus, reducing their economic value as ornamental plants (Hosagoudar et al., 1997). However, these fungi are not known to cause significant damage to crops (Hosagoudar, 2003).

Hyperparasitic fungi of several genera, mainly belonging to Dothideomycetes or Sordariomycetes, have been reported on species of Meliolales (Deighton and Pirozynski, 1972). These hyperparasites frequently overgrow the entire colonies of the black mildews until the presence of the meliolalean host may be proved only by careful search under a light microscope. Several species of hyperparasitic fungi may be found on the same leaf and even on the same colony (Stevens, 1918; Ciferri, 1955).

Information about fungal hyperparasites on species of Meliolales is scattered throughout literature and no exact number of known species has been reported to date. Most species have been described based on morphology before the widespread use of molecular techniques in fungal taxonomy. Therefore, the modern systematic position of many species of hyperparasitic fungi is unknown. In this review, we compile information available on fungi that parasitize colonies of Meliolales, to highlight knowledge gaps and to aid conceptualization of future research projects.

2. HYPERPARASITIC FUNGI ON MELIOLALES: LITERATURE REVIEW

2.1. Mode of Host Interaction

Hyperparasitic fungi are classified into two groups based on the mode of parasitism and the effects on the fungal host: necrotrophic and biotrophic parasites (Boosalis, 1964; Barnett and Binder, 1973; Jeffries, 1995; Benjamin et al., 2004; Sun et al., 2019). Necrotrophic hyperparasites invade and kill their fungal hosts, while biotrophic hyperparasites take nutrients from living cells of the fungal host (Jeffries, 1995; Moore et al., 2020). The relationship between the biotrophic parasite and the fungal host is physiologically balanced. The cytoplasm of the host remains functional (Jeffries, 1995). Depending on the type of interaction, i.e., the parasite-host interface, biotrophic hyperparasites are classified into three groups (Barnett and Binder, 1973; Jeffries, 1995; Sun et al., 2019; Moore et al., 2020):

- **Intracellular biotrophs.** Hyphae of the hyperparasite enter the cells of the host fungus.
- **Haustorial biotrophs.** Parts of cells of the hyperparasite penetrate into cells of the host fungus and form haustoria for nutrient uptake.
- **Contact/fusion biotrophs.** Cells of the hyperparasite are in close contact and/or fuse with the cells of the host fungus.

Most fungi that grow on colonies of Meliolales are obligate biotrophs, as they are found in the field only together with the parasitic host, and there is no history of cultivation on artificial media. Based on morphological and physiological observations only (Jeffries, 1995), nutrients are possibly transferred *via* the interface. In fact, our microscopic observations of material from Panama (**Figure 2**) indicate that these fungi establish an intimate contact with the hyphae of the host (contact/fusion biotrophs) without the presence of haustoria.

Hyperparasitic interactions are difficult to prove but may be assumed when the parasite causes distinctive morphological or physiological alterations in the host (Jeffries, 1995). In the case of species of Meliolales, hyperparasitic fungi may overgrow their colonies, and prevent the black mildew fungus from producing spores and ascomata (Stevens, 1918; Toro, 1952). Hyperparasites also modify some vegetative structures of Meliolales, such as the density and branching of hyphae, the number, shape or size of hyphopodia, and the presence, number, disposition, size, and shape of setae (Ciferri, 1955). This antagonistic activity and the
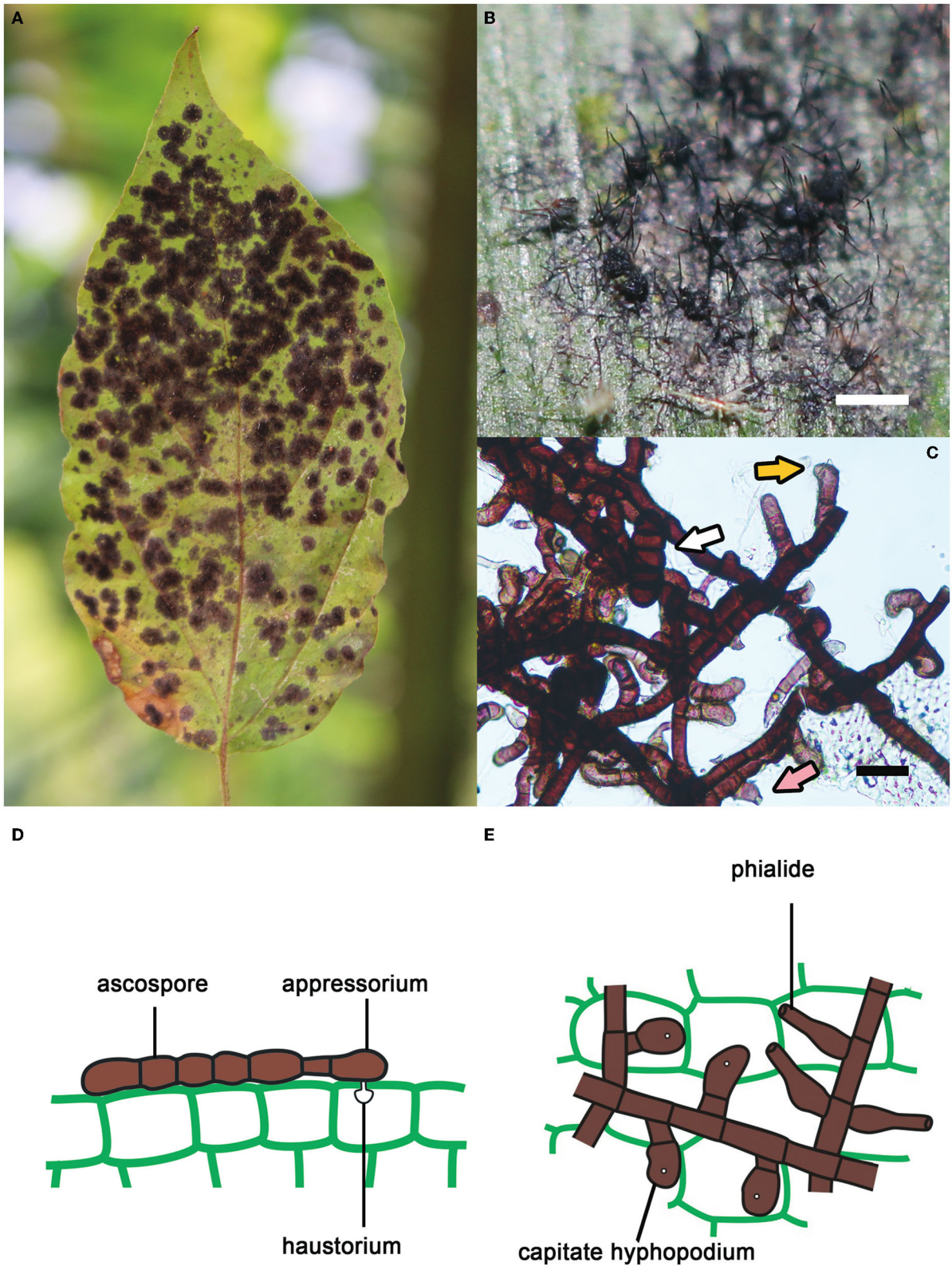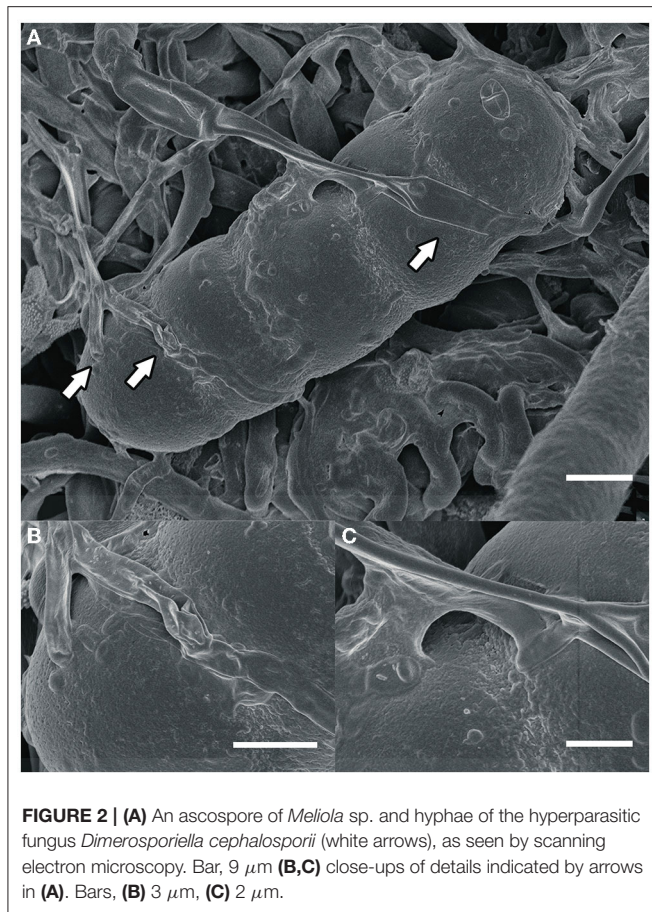
FIGURE 1 | Key features of species of Meliolales. **(A)** Black colonies of *Meliola clerodendricola* on a leaf of *Clerodendrum* sp. **(B)** Superficial hyphae, perithecia and setae of *Meliola* sp. on a leaf of *Olyra latifolia*. Bar, 1 mm. **(C)** Hyphae of *Meliola mangiferae* with capitate hyphopodia (yellow arrow), a phialide (pink arrow) and a septate ascospore (white arrow). Bar, 20 μm. **(D,E)** Schematic drawings of cells of *Meliola* spp. **(D)** Ascospore on the surface of host tissue with a capitate hyphopodium including an appressorium penetrating the wall of the epidermis. **(E)** Hyphae with capitate hyphopodia and phialides.

FIGURE 2 | (A) An ascospore of *Meliola* sp. and hyphae of the hyperparasitic fungus *Dimerosporiella cephalosporii* (white arrows), as seen by scanning electron microscopy. Bar, 9 μm **(B,C)** close-ups of details indicated by arrows in **(A)**. Bars, **(B)** 3 μm, **(C)** 2 μm.

incapability of hyperparasitic fungi to grow on artificial media strongly suggests that they are obligate parasites (Jeffries, 1995).

2.2. Morphological Classification

Hyperparasitic fungi form an ecological guild and include organisms from diverse taxonomic groups. Systematic positions of these fungi are mostly unknown and they are poorly represented in sequence databases. Here we use traditional morphological categories to classify 189 species of hyperparasitic fungi that grow on colonies of species of Meliolales (**Supplementary Material 1**): dematiaceous hyphomycetes, moniliaceous hyphomycetes, pycnidioid, perithecioid, catathecioid, and apothecioid fungi (**Figure 3**).

2.2.1. Dematiaceous Hyphomycetes (21 Genera, 40 Species)

The artificial group of "dematiaceous" or "dark hyphomycetes" comprises conidial fungi that have heavily melanized, brown-pigmented hyphae and do not form fruiting bodies (Revankar and Sutton, 2010). All genera within this group comprise hyperparasitic species as well as fungi parasitic of other fungi and plants.

Atractilina parasitica, one of the most common hyperparasites of Meliolales, form distinctive straw colored synnemata which are composed of aggregated conidiophores (**Figure 4A**). This

fungus grows almost exclusively on black mildew hosts and has been reported mostly for Africa (Deighton and Pirozynski, 1972). Other common hyperparasitic dematiaceous fungi of black mildews are species of *Helminthosporium* and *Spiropes*. In the past, they were sometimes considered to correspond to conidial stages of species of Meliolales (Ciferri, 1955).

2.2.2. Moniliaceous Hyphomycetes (30 Genera, 52 Species)

Conidial fungi without fruiting bodies and not or only slightly pigmented cells are grouped as moniliaceous hyphomycetes. Some species in this group are only known on black mildews, e.g., *Acremoniula suprameliola*, *Chionomyces chorleyi*, *Chionomyces meliolicola*, *Eriomycopsis biseptata*, *Trichoconis hamata*, as well as the following four species representing monotypic genera: *Divinia diatricha*, *Monosporiella meliolicola*, *Spermatoloncha maticola*, and *Tuberculispora jamaicensis* (Hansford, 1942; Hawksworth, 1981). Other species of hyperparasitic moniliaceous hyphomycetes are more flexible concerning their fungal host range.

2.2.3. Pycnidioid Fungi (5 Genera, 10 Species)

Species of five genera of asexual fungi forming conidia in pycnidia have been reported as parasites of black mildews, namely *Capitorostrum*, *Chaetophoma*, *Cicinnobella*, *Coniothyrium*, and *Naemosphaera* (Stevens, 1918; Petrak, 1950; Hawksworth, 1981). These genera also comprise species parasitic on plants or on other fungi.

2.2.4. Perithecioid Fungi (23 Genera, 68 Species)

Perithecioid hyperparasites develop perithecia containing asci to produce spores. Many genera of this group have been revised by Batista and da Silva (1960), Pirozynski (1977), Rossman (1987), and Rossman et al. (1999). Some examples are the bitunicate ascomycetes of the genera *Paranectriella* and *Puttemansia*, and species with unitunicate asci in *Nematothecium* and *Rizalia*. *Dimerosporiella cephalosporii* (**Figure 4B**) is one of the most common parasites of *Meliola* spp. in the tropics (Gams et al., 2004). All 11 species of the genus *Melioliphila* are parasites specifically of colonies of black mildews.

2.2.5. Catathecioid Fungi (1 Genus, 17 Species)

Species of the genus *Trichothyrium* are strictly hyperparasitic, and they grow on colonies of Asterinales, Meliolales and other foliicolous species of Ascomycota (Piepenbring, 2015). They are characterized by the presence of catathecia, i.e., flattened perithecia with a well-developed upper and lower peridial wall, and densely packed hyphae that form bands covering hyphae of the host fungus. The delimitation of species in this genus is ambiguous, as only a few morphological characteristics are used, such as the size of ascospores (Wu et al., 2011; Hongsanan et al., 2020).

2.2.6. Apothecioid Fungi (2 Genera, 2 Species)

Two species of fungi with apothecia are known as hyperparasitic fungi on Meliolales. The genus *Unguiculella* comprises mostly saprotrophic species, and *U. meliolicola* is the only species in this genus known to be parasitic on black mildews (Dennis,
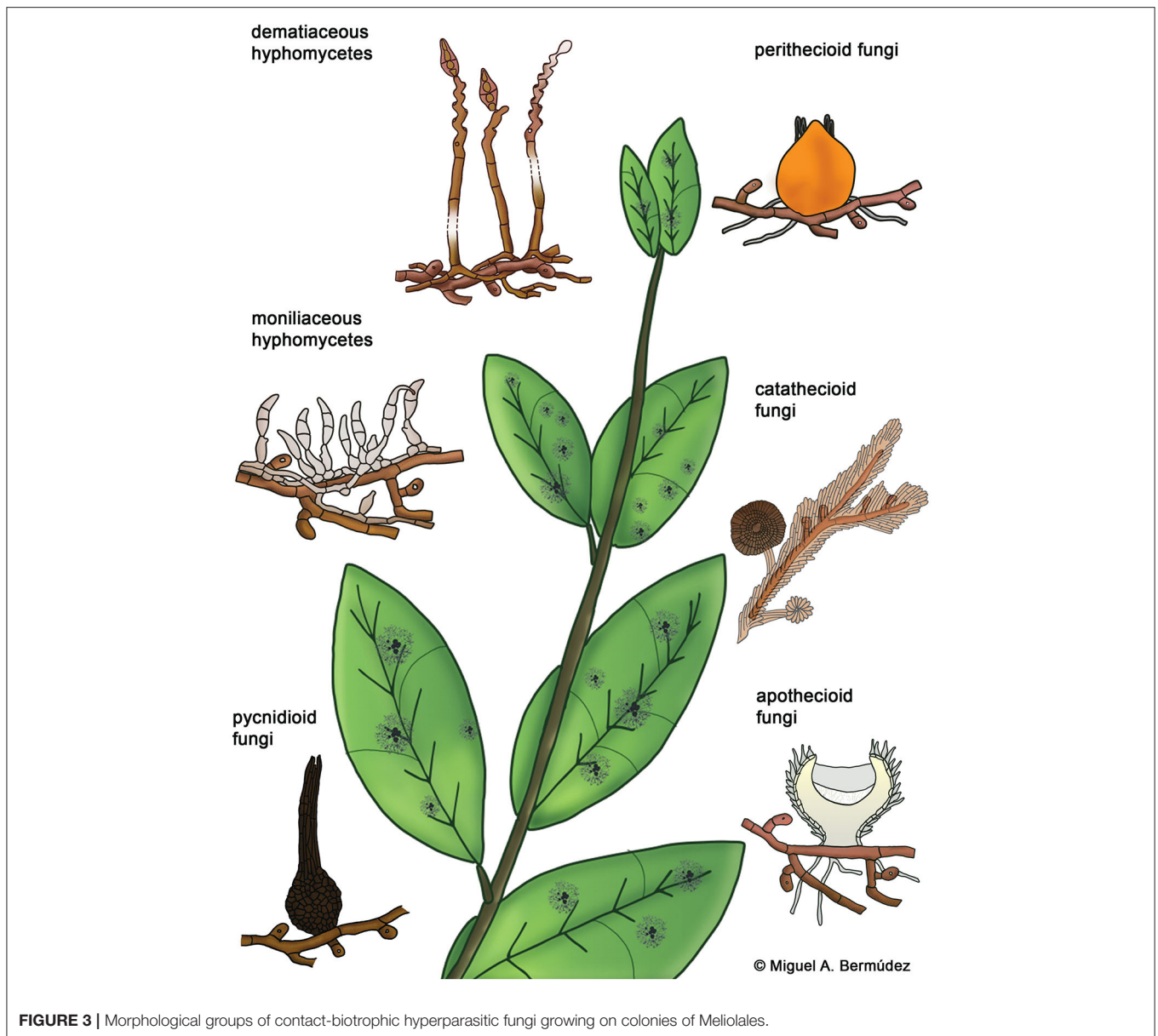
FIGURE 3 | Morphological groups of contact-biotrophic hyperparasitic fungi growing on colonies of Meliolales.

1955). The situation for the genus *Calloriopsis* is similar with *C. herpotricha* being the only species hyperparasitic on Meliolales (Sydow and Sydow, 1917, cited as *C. gelatinosa*).

2.3. Ecology of Hyperparasitic Interactions

Hyperparasitic fungi may shape the dynamics of the interaction between the plant host and the host fungus, increase the complexity of the food webs and play a significant role in regulating population sizes (Gleason et al., 2014; Sandhu et al., 2021). Hyperparasitic fungi decrease the fitness of the host fungus by inducing hypovirulence and increasing its death rate, eventually clearing the parasitic infection and leading to an uninfected host (Northrup et al., 2021; Sandhu et al., 2021). These effects, to some extent, exert a positive effect on the fitness of host plants and may be used in the context of biocontrol (Kiss, 2001).

In the specific case of Meliolales, the population ecology of the host fungus is affected by decreased sporulation (Jeffries, 1995). This limits the dispersal and extension rates of the plant-parasitic fungus. However, Hawksworth (1981) observed that the largest colonies of black mildews are often the richest in hyperparasitic fungi, suggesting that the hyperparasitic fungi may not be really harmful. More in-depth studies on the ecology of these organisms are necessary in order to understand the type of interaction they have with their hosts.

The surface of the setae and of other cells of meliolalean fungi is hydrophilic, thus the colony is easily wetted. This characteristic results in a prolonged state of moisture of the colony and allows the growth of other organisms that use this specific niche. We observed algae, like *Cephaleuros virescens*, yeasts, cyanobacteria, other bacteria and small animals,
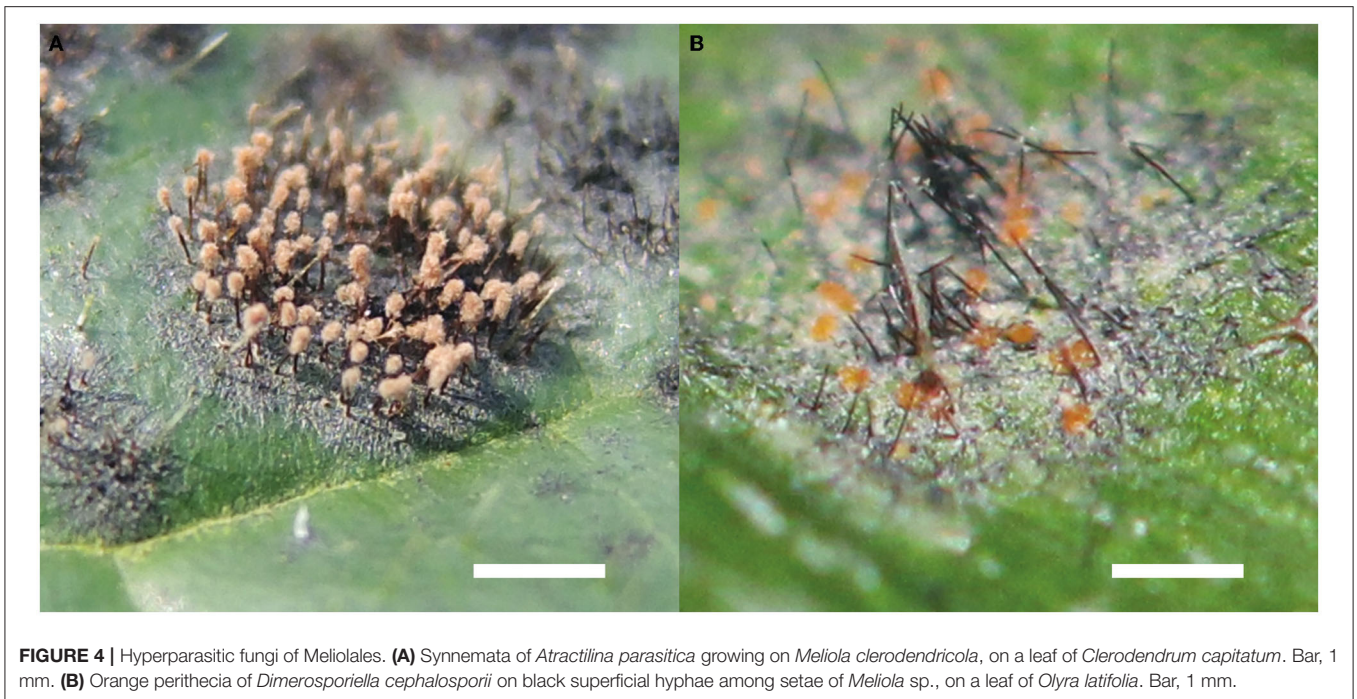
FIGURE 4 | Hyperparasitic fungi of Meliolales. **(A)** Synnemata of *Atractilina parasitica* growing on *Meliola clerodendricola*, on a leaf of *Clerodendrum capitatum*. Bar, 1 mm. **(B)** Orange perithecia of *Dimerosporiella cephalosporii* on black superficial hyphae among setae of *Meliola* sp., on a leaf of *Olyra latifolia*. Bar, 1 mm.

like mites and tardigrades, in the colonies of black mildews. Metabolites excreted by these organisms and the nitrogen fixed by cyanobacteria may serve as sources of nutrients for Meliolales and may promote the growth of hyperparasites (Piepenbring et al., 2011; Piepenbring, 2015). According to Kiss (2001), a hyperparasitic interaction consists of three trophic levels, but interactions between plants, plant parasites, hyperparasites and these other organisms are certainly more diverse and complex than these three levels indicate.

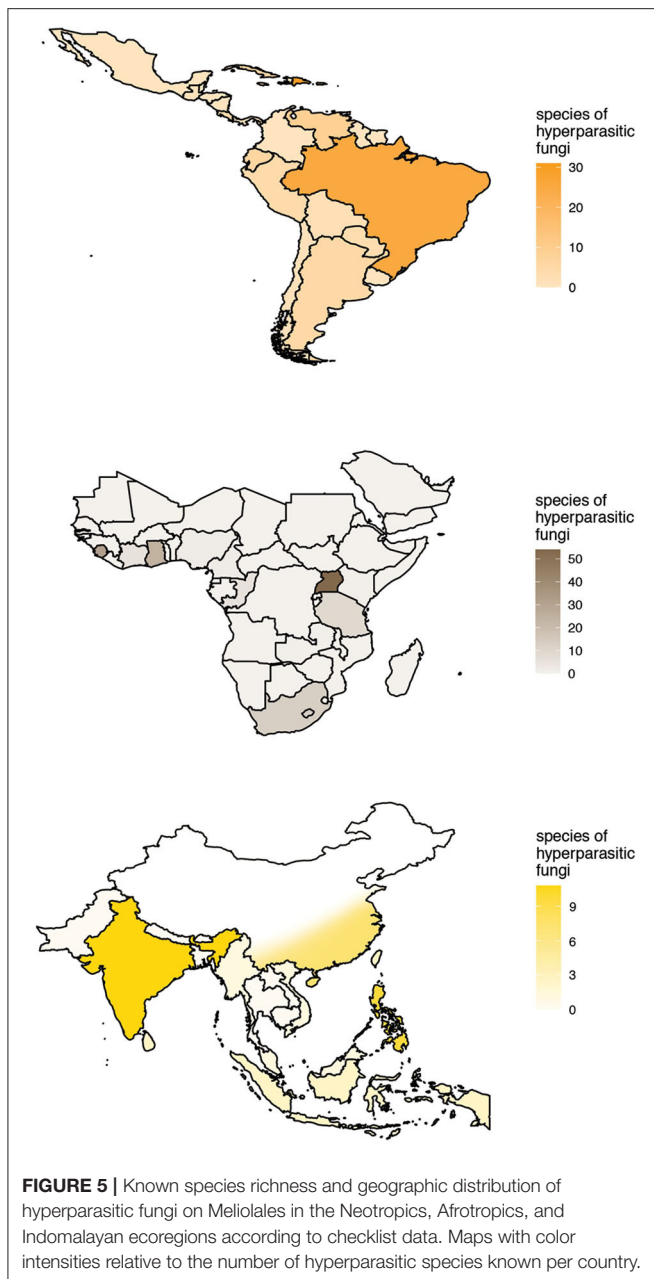## 2.4. Analysis of the Species Checklist: Evidencing the Gaps of Knowledge
2.4.1. Species Richness of Hyperparasitic Fungi
To date, no precise number of species of fungi parasitizing black mildew exists. Gams et al. (2004) estimated approximately 75 species of fungi parasitic on black mildews and other leaf-inhabiting fungi. A similar number is found in the species checklist presented by Sun et al. (2019): among 1552 species of fungicolous fungi, i.e., fungi that grow on other fungi that are not necessarily parasitic, 78 species of hyperparasites on Meliolales are reported.

The checklist of hyperparasitic fungi growing on Meliolales presented here is based on primary literature, i.e., scientific publications in international journals with peer review process, and books with ISBN number, as well as secondary literature like review papers, databases, and lists. The publications were found in Google Scholar, Cybertruffle (Minter, 2020), Biodiversity Heritage Library (Gwinn and Rinaldo, 2009), and by references in the analyzed publications. A list with information on type data of species of hyperparasitic fungi on black mildews was obtained from data compiled in Index Fungorum (Kirk, 2019).

The checklist (**Supplementary Material 1**) contains information for records of hyperparasitic fungi growing on Meliolales in an Excel file, including valid scientific names; systematic positions; names of fungal and plant hosts; family of plant hosts; morphological classification; synonyms according to Index Fungorum, MycoBank (Crous et al., 2004), and Zeng et al. (2022); geographic distribution; and references (see **Supplementary Material 2**). Data analyses were performed with R v4.1.2 (R Core Team, 2022). The package maps v3.4.0 (Becker et al., 2021) was used to draw maps of the different ecoregions, and functions in the package vegan v2.5-7 (Oksanen et al., 2020) were used to build curves of species accumulation with sampling covering, based on the number of records. An R script modified from Piepenbring et al. (2020) was also used for the analyses of the checklist data. Synonyms were no included in the analyses.
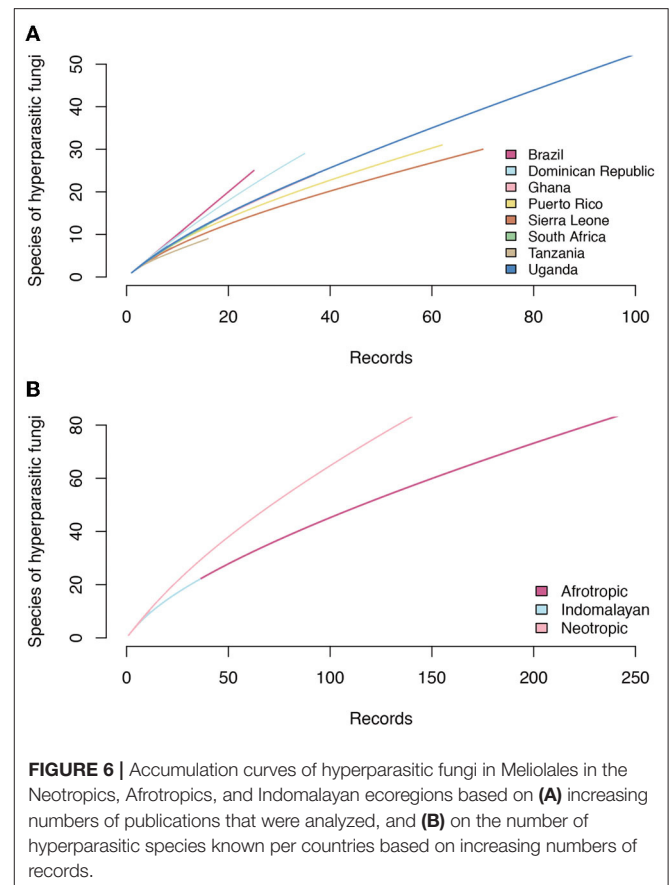
The checklist contains 525 records of hyperparasitic fungi known from all over the world. These refer to 189 species of hyperparasitic fungi growing on colonies of Meliolales, comprised in 82 genera. Thereby, we report more than twice as many hyperparasitic species as cited by other authors up to now. Records were retrieved from 86 publications (**Supplementary Material 2**). The number of known species of hyperparasitic fungi is maximal in the afrotropics for Uganda (54), followed by Sierra Leone (31) and Ghana (24). In the neotropics, 31 species of hyperparasitic fungi are reported for Puerto Rico, 30 for the Dominican Republic and 25 for Brazil; and in the indomalayan ecoregion, nine and eight species have been reported for India and the Philippines, respectively. The geographic distribution of the species richness known per country is plotted in **Figure 5**, with color intensities relative to the number of species known per country. Only the most species-rich ecoregions are shown in the graphs.

FIGURE 5 | Known species richness and geographic distribution of hyperparasitic fungi on Meliolales in the Neotropics, Afrotropics, and Indomalayan ecoregions according to checklist data. Maps with color intensities relative to the number of hyperparasitic species known per country.

FIGURE 6 | Accumulation curves of hyperparasitic fungi in Meliolales in the Neotropics, Afrotropics, and Indomalayan ecoregions based on **(A)** increasing numbers of publications that were analyzed, and **(B)** on the number of hyperparasitic species known per countries based on increasing numbers of records.

- Some relevant publications were not available for analysis, as they are hidden in old, local and/or inaccessible journals.
- Identifications of species of hyperparasites and parasites published in literature may not be correct.
- As the species have only been described morphologically, the systematic position of hyperparasites is not resolved and the delimitation of most genera is not well known.

2.4.2. History of Description of Hyperparasitic Fungi

The first scientific investigation of hyperparasitic fungi growing on colonies of Meliolales started in the 1800s with the work of Carlo Spegazzini (Spegazzini, 1889) through an inventory of fungal species in Patagonia, Argentina. The oldest name of a hyperparasitic fungus of Meliolales is *Peziza herpotricha* Berk. (current name: *Calloriopsis herpotricha*), which, however, was not recognized as a hyperparasite by Berkeley (Hooker, 1851). In the following years, only few reports of hyperparasitic fungi are mentioned mainly in publications dealing with individual groups of fungi, or in species inventories (e.g., Patouillard, 1892; Hennings, 1904; Sydow and Sydow, 1917). Some publications center around hyperparasitic fungi on different hosts (primary literature: e.g., Hansford, 1946; Batista et al., 1966; Deighton and Pirozynski, 1972; Pirozynski, 1977; Katumoto, 1987; review papers: Hawksworth, 1981; Gams et al., 2004; Sun et al., 2019), and only a few publications focus specifically on

Accumulation curves for hyperparasitic fungal species known for the neotropics, the afrotropics and the Indomalayan region do not reach saturation for any country (**Figure 6**). Thus, sampling and documentation of the diversity of hyperparasitic fungi in the tropics and subtropics is still incomplete.

The records in the checklist were extracted from literature and adjusted to the checklist concept to the best of our knowledge. Nevertheless, the checklist is still incomplete and some information may not be correct due to the following reasons.
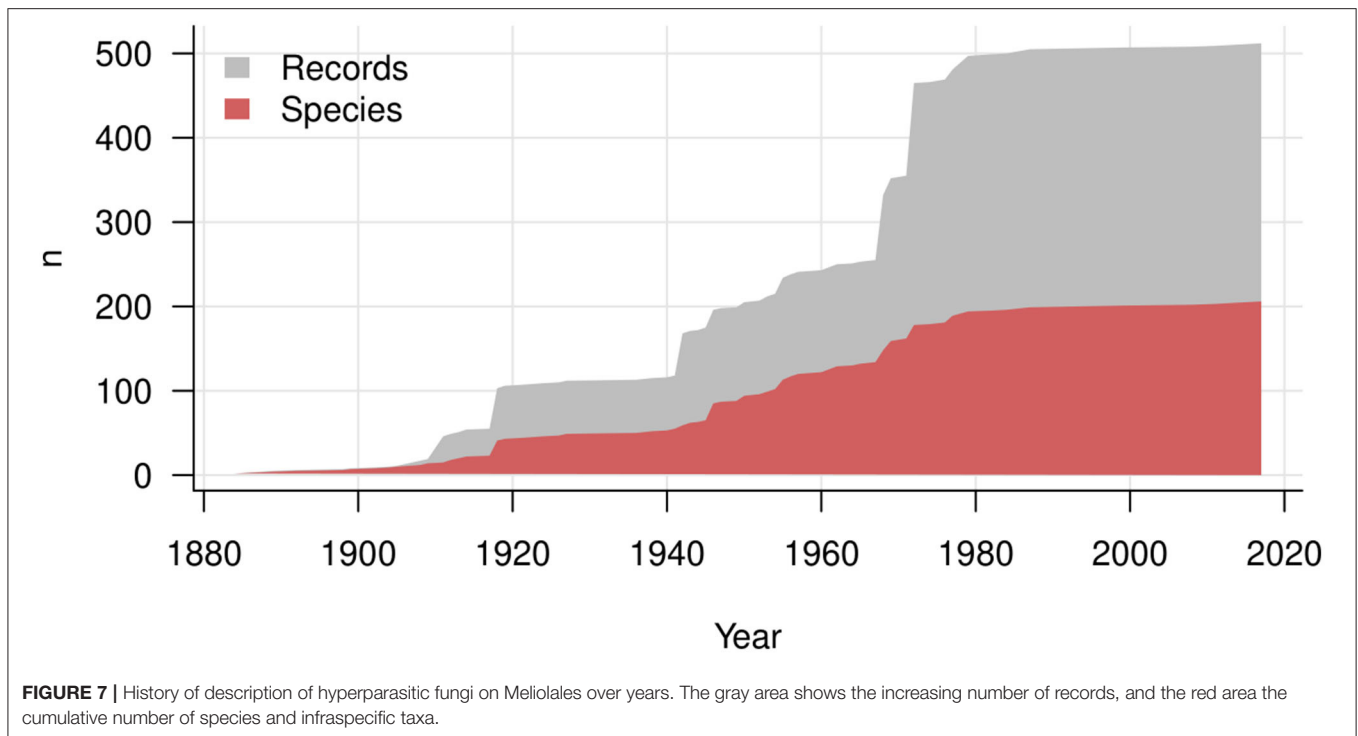
- As the information on hyperparasitic fungi on Meliolales is scattered through literature, it is very likely that further records of hyperparasitic species are hidden in literature.

FIGURE 7 | History of description of hyperparasitic fungi on Meliolales over years. The gray area shows the increasing number of records, and the red area the cumulative number of species and infraspecific taxa.

Meliolales and their parasites (Stevens, 1918; Ciferri, 1955; Farr, 1969).

Major contributions are exhibited as jumps in the accumulation lines of records in **Figure 7**. These contributions include publications by Stevens (1918: 14 species reported for Puerto Rico), Hansford (1946: 17 species reported mostly for Uganda and Ghana), Ellis (1968: 13 species of the genus *Spiropes*) and Deighton and Pirozynski (1972: 16 species reported mostly for Africa). The corresponding jumps are lower than the numbers of records, because many species were reported more than once. As a result, the total number of records has increased much more rapidly than the total number of known species since the 1980s. A plateau of the curves of records and species indicates that hyperparasites on Meliolales were not investigated during the last 20 years, except for one new species combination, *Trichothyrium peristomale*, proposed by Wu et al. (2011). Most current studies on hyperparasitic fungi have focused on their use in biocontrol experiments, which are directed toward reducing the damage caused by a plant pathogen (Day, 2002). Meliolales and their hyperparasites are not aggressive parasites, thus researchers have focused on hyperparasites that cause high mortality of a primary parasite, e.g., hyperparasites on rust fungi.

2.4.3. Systematic Position of Hyperparasitic Species

All 189 taxa in the checklist (**Supplementary Material 1**) are species of Ascomycota. Among them, a total of 110 species are "incertae sedis" ("uncertain position") for one or several levels of classification. For 61 species, the systematic position at class level is unknown; for 106 species, the systematic position at order level is unknown, and for 67 species, the systematic position at family level is unknown.

Some conidial forms, especially dematiaceous and moniliaceous hyphomycetes may represent anamorphic stages of certain teleomorphic hyperparasitic fungi that grow on colonies of species of Meliolales. *Dimerosporiella cephalosporii*, for example, is usually found together with an *Acremonium*-like anamorph (Gams et al., 2004). Species of the genus *Isthmospora* are considered as conidial stages of *Trichothyrium* spp. (Ciferri, 1955). Conidia of these hyphomycetes, however, may also be found without perithecia or catathecia. To date, the anamorph-teleomorph connection of many hyperparasitic fungi remains elusive, and it is difficult to determine the precise number of species.

Concepts of genera are based on morphological characteristics and on short Latin descriptions. Fresh collections and DNA sequence data are necessary to establish natural concepts of genera and to elucidate their systematic position. Molecular investigation may also provide evidence on further anamorph-teleomorph connections.

2.5. Network Analysis of Host Ranges of Hyperparasitic Fungi

To document and understand the diversity and specificity of species interactions, network theory is frequently used in ecological research. Species are represented as units (nodes) that form interactions (links). This approach serves to visualize species interactions (Pocock et al., 2016) or to characterize the structure of ecological communities (Dormann et al., 2009). Most studies on tritrophic parasitic networks (in the wider sense) were conducted on phytophagous insects and their insect parasites or parasitoids that infect them (Derocles et al., 2018; de Araujo and
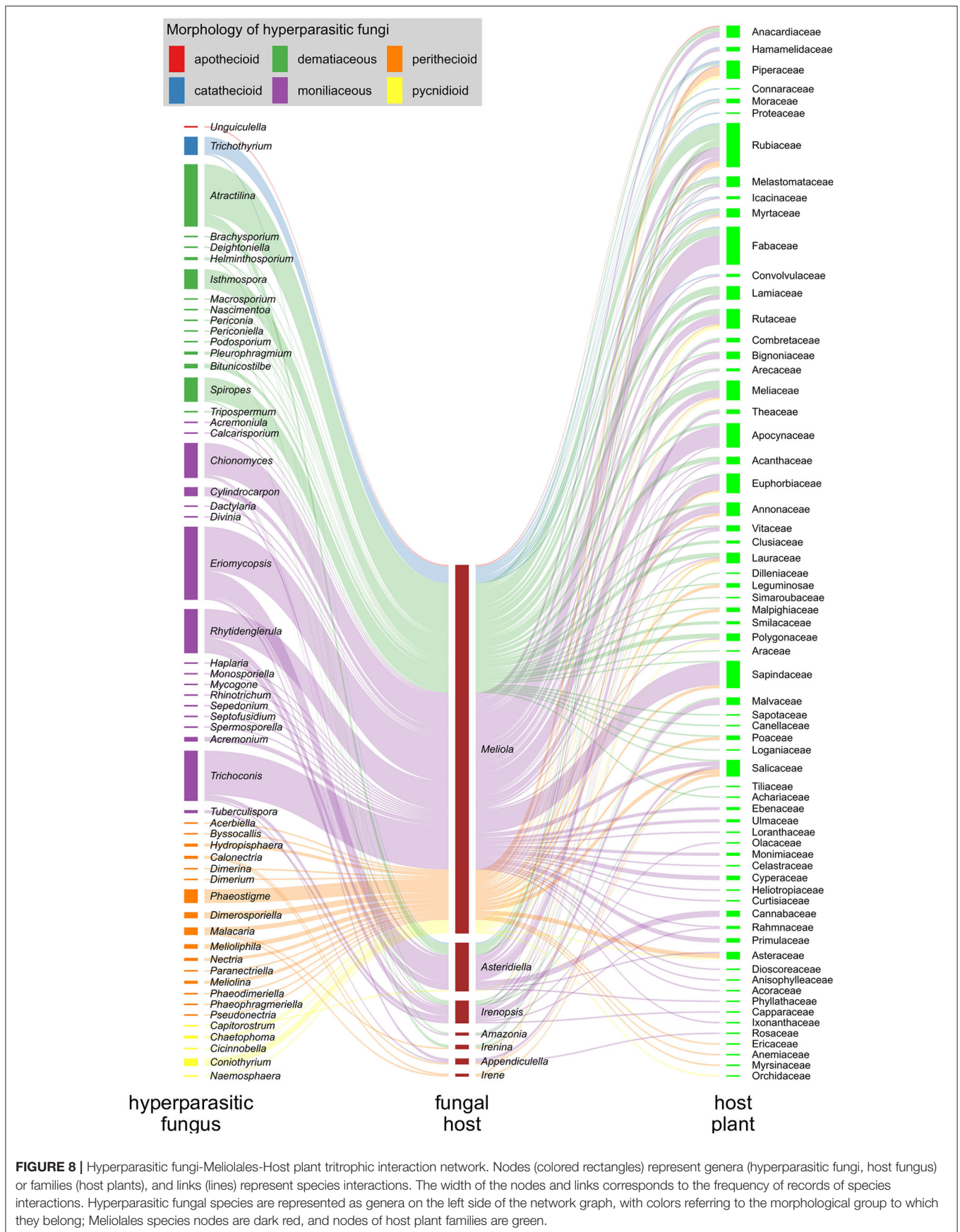
FIGURE 8 | Hyperparasitic fungi-Meliolales-Host plant tritrophic interaction network. Nodes (colored rectangles) represent genera (hyperparasitic fungi, host fungus) or families (host plants), and links (lines) represent species interactions. The width of the nodes and links corresponds to the frequency of records of species interactions. Hyperparasitic fungal species are represented as genera on the left side of the network graph, with colors referring to the morphological group to which they belong; Meliolales species nodes are dark red, and nodes of host plant families are green.

Maia, 2021; Kawatsu et al., 2021). Network theory has not yet been applied to fungal hyperparasitic-host fungus interactions.

In **Figure 8**, we illustrate the interactions of hyperparasitic fungi infecting species of Meliolales, which are themselves parasitic on plants (**Supplementary Material 1**), in a network. Fungal hyperparasites and their fungal hosts are grouped by genus, and their plant hosts by family. Hyperparasitic interactions with fungal and plant hosts not identified to genus and family level respectively were excluded. The network was visualized using the packages ggforce v0.3.3 (Pedersen, 2021) and ggplot2 v3.3.5 (Wickham, 2016) in R v4.1.2 (R Core Team, 2022). Colors were used to highlight morphological groups of hyperparasitic fungi.

The graph is based on 300 records of species of hyperparasitic fungi that were found on different genera of Meliolales. Moniliaceous hyphomycetes were observed most frequently, followed by dematiaceous hyphomycetes and perithecioid fungi (**Figure 8**). The abundance of genera of Meliolales reflects the abundance and known species richness of genera of Meliolales, with *Meliola* being be far the most frequent and species rich genus. The abundance of plant host families reflects known host preferences of species of Meliolales among species of angiosperms, with Apocynaceae, Euphorbiaceae, Fabaceae, Rubiaceae, and Sapindaceae, presenting an elevated number of species of Meliolales.

The host range of most genera of hyperparasitic fungi includes several species of one or several genera of Meliolales, i.e., hyperparasitic fungi are generalists concerning their hosts among Meliolales. The network graph shows a notorious preference of most species of hyperparasitic fungi for species of the genus *Meliola*, independently of the generic position and the morphological group of the hyperparasitic fungus. As a genus with diverse and abundant host species, the chances of *Meliola* spp. being colonized by hyperparasitic fungi are higher than for species of other genera of Meliolales. According to Vazquez et al. (2005), for host-parasite systems, the more abundant host taxa tend to have a higher diversity of parasites and to have a higher representation of specialist parasites.

The association between hyperparasitic fungi and host plants is diverse and aleatory, and no correlation between both groups is observed. Host plant diversity does not depend on the hyperparasites but on the host fungi (Meliolales), that are known to be host specific at the level of species, genera, or families (Jayawardena et al., 2020).

Concerning the conclusions drawn from this analysis, several important aspects need to be considered.

- For 31 species of hyperparasitic fungi, associations are represented only by a single specimen. In this case, a single connection is shown in the graph, suggesting that these species of hyperparasitic fungi are highly specific. This is most likely not the case, when sampling efforts are increased.
- In addition to the susceptibility of the host fungi, numerous further factors are important for the occurrence of parasite-hyperparasite interactions, especially environmental conditions (Bryner and Rigling, 2011; Kohl et al., 2019) and the availability of inoculum. There are no data available to further discuss these aspects.

- Genera of Meliolales are based on morphological characteristics and preliminary sequence data shows that new circumscriptions and placement of genera will be required (Mibey and Hawksworth, 1997; Marasinghe et al., 2020; Jayawardena et al., 2021; Zeng et al., 2022). The non-specificity between groups of hyperparasitic fungi and genera of Meliolales may be a consequence of the fact that meliolalean genera are artificial. We do not expect, however, to see host specificity even with natural genera.

Beyond data presented in this network analysis, it is important to mention that not all species of hyperparasitic fungi are restricted to meliolalean hosts. *Eriomycopsis flagellata*, for example, parasitizes colonies of *Asteridiella* and *Meliola* (Meliolales), *Asterina* (Asterinales), and *Balladyna* (Balladynaceae). Nevertheless, literature research and our sampling experience indicate that most species of hyperparasitic fungi are restricted to meliolalean hosts.

In the case of species of hyperparasitic fungi for which several records are available, broad host spectra are observed. For *Eriomycopsis bomplandi*, for example, 27 records are available, referring to 22 different host species. Apparently, hyperparasitic fungi are generalists not only at the genus level, but also at the species level of the host fungus.

Building multitrophic ecological networks is a difficult task, especially in poorly studied and highly diverse systems (Derocles et al., 2018), as is the case for hyperparasitic fungi and black mildews. Sampling efforts need to be increased and data from more countries and host fungi should be included to strengthen future analyses of these species' interactions (Cazabonne et al., 2022).

2.6. Problems Related to Molecular Sequencing of Hyperparasitic Fungi

To date, no sequencing data are available for any fungal species hyperparasitic on Meliolales. Here we present some reasons that might have prevented the development of methods for molecular studies of these organisms:

a. Strong melanization. Melanin is a ubiquitous compound that is present in many fungal cell walls with varying quantities depending on the species (Revankar and Sutton, 2010). For example, species of the genus *Spiropes*, common hyperparasites of Meliolales, have a tough surface layer of melanin in their cell walls. This inert polymer is insoluble at cold temperatures and impermeable to boiling and organic solvents. Melanin is also highly resistant to UV light, acids, and enzymatic digestion (Karakousis et al., 2006). According to Eckhart et al. (2000), melanin is also a potent inhibitor of thermostable DNA polymerase, and the inhibitory effect is conferred by a direct and reversible polymerase-melanin interaction.

b. Biomass. The reproductive structures of hyperparasitic microfungi, when present, are less than 1 mm in size and are present in limited quantities. This makes the extraction procedures difficult, as many DNA extraction methods depend on adequate biomass of the organism.

c. Mixed-infections. Isolating DNA from only one specific hyperparasite without contamination by other organisms

remains challenging. DNA sequences resulting from these samples might be attributed to the wrong species.

d. The lack of DNA sequences for comparison. As there are no DNA sequences available for any species of mycoparasites of Meliolales, no reference sequences exist. Apparently most hyperparasitic fungi of black mildews are obligate biotrophs and cannot be grown separate from their hosts. The hosts themselves are also biotrophic parasites making it challenging to isolate and sequence the hyperparasites.

e. No single method. Hyperparasitic fungi have different morphologies and belong to diverse systematic relationships. Therefore, the molecular methods to study them may vary depending on each group.

The development of methods to study the DNA of hyperparasitic microfungi is a necessary task in order to better understand the diversity and evolution of this guild of fungi.

3. DISCUSSION

By the present contribution, information on species of fungi hyperparasitic on Meliolales is compiled in a checklist for the first time. Checklists on species diversity are essential sources of information for the characterization of biodiversity in any given area. These lists help to understand the present state of knowledge of fungi in the area and provide information on the ecology, taxonomy and biogeography of fungi, especially of undersampled taxonomic and ecological groups (Piepenbring et al., 2020). The determination of fungi in the tropics is a great challenge due to the lack of monographs, reference specimens and expertise (Piepenbring et al., 2018). Moreover, there is no detailed treatment of biotrophic plant pathogens and their parasites (Gams et al., 2004) as most publications deal with individual groups of fungi.

The huge diversity of reproductive structures presented by hyperparasitic fungi on Meliolales indicates the polyphyletic nature of this ecological group. Colonies of Meliolales were "discovered" repeatedly during evolution by fungi belonging to different systematic groups.

In the context of the present study, a tritrophic network analysis of fungi hyperparasitic on plant-parasitic fungi is presented for the first time. Hyperparasitic fungi are generalists concerning genera of Meliolales. This can be explained by

the fact that they are contact parasites and do not penetrate into host cells. However, most species of hyperparasitic fungi are specific to Meliolales, probably due to the specific growth conditions provided by the meliolalean colonies, i.e., moisture and metabolites of associated microorganisms.

As meliolalean fungi and their hyperparasites are not aggressive parasites, they are not in the focus of applied mycological research. However, we need further morphological, molecular and ecological studies on these fungi in order to understand their diversity, evolution and biology.

AUTHOR CONTRIBUTIONS

MB-C compiled and analyzed the data and wrote the first draft. AC-L performed the network analysis. MB-C, AC-L, and MP contributed to writing and editing the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/ffunb.2022.885279/full#supplementary-material

Supplementary Material 1 | Checklist of hyperparasitic fungi on Meliolales known for the tropics and subtropics based on literature.

Supplementary Material 2 | References to literature containing records of hyperparasitic fungi on Meliolales for tropical and subtropical countries and cited in **Supplementary Material 1**.

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Anlage 2

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Hyperparasitic fungi—definitions, diversity, ecology, and research

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Abstract

Even parasites have parasites. By definition, a hyperparasite is an organism capable of parasitizing another parasite. Hyperparasitism caused by fungi is a common phenomenon in nature, but it has been poorly studied. This life history strategy evolved several times in the fungal tree of life, and is crucial in the maintenance of ecosystems as well as in the mediation of parasite–host interactions. Although the interest for hyperparasitic fungi is growing in the context of biological control, hyperparasitism is not ecologically and evolutionarily understood. This chapter summarizes the most relevant aspects of the terminology, diversity, and ecology of hyperparasitic fungi on both fungal and non-fungal hosts. We also discuss the problems related to molecular research on hyperparasitic fungi. As they represent a hidden source of diversity, it is necessary to increase sampling efforts and to undertake further morphological, molecular, and ecological studies to understand these fungi and their potential biotechnological and pharmaceutical uses.

1. Hyperparasitism

All living organisms can take part in parasitic relationships, either as parasites or as hosts (Combes, 2001; Krasylenko *et al.*, 2021). Interactions between parasites and their hosts are typically regarded as closed one-to-one systems. In reality, however, these relationships involve complex multitrophic interactions (Kiss, 2001). The term "hyperparasite" refers to an organism that parasitizes another parasitic organism (**Fig. 1**). Hyperparasitism has been well documented for many groups of organisms, mainly insect parasitoids associated with parasitoid hosts, viruses that parasitize disease-causing protozoans, and parasitic flowering plants (Grybchuk *et al.*, 2018; Krasylenko *et al.*, 2021; Sullivan, 1987). Hyperparasitism by fungi is poorly studied, even though it is thought to be rather widespread in nature (Haelewaters *et al.*, 2018a, 2021a; Parratt and Laine, 2016; Sun *et al.*, 2019). As fungi are able to parasitize organisms from different kingdoms (Moore *et al.*, 2020), this chapter focuses on fungal hyperparasites parasitic on both fungal and non-fungal hosts.
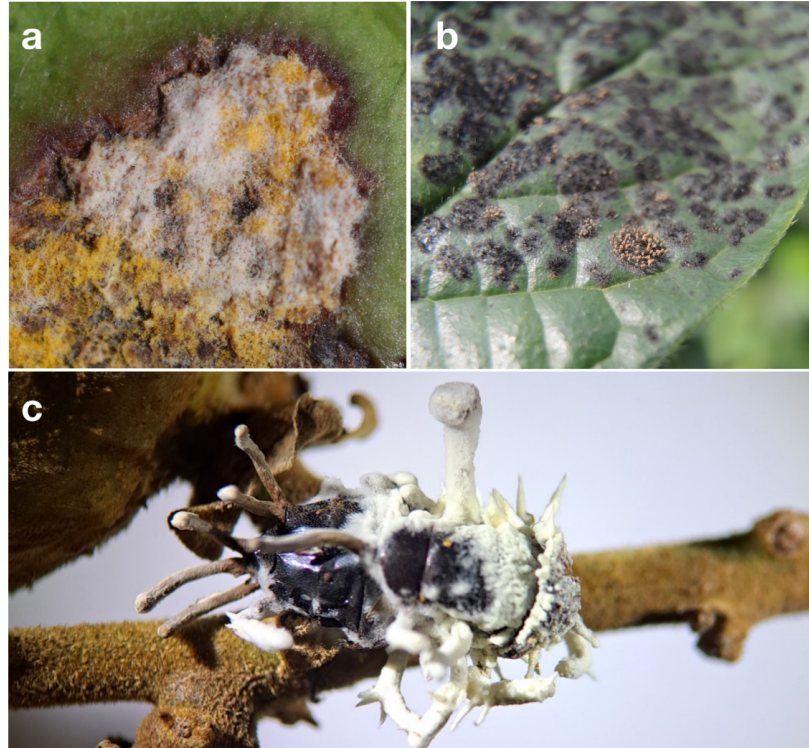
Figure 1: Examples of hyperparasitic fungi. a. The fungus Akanthomyces lecanii (white) growing on a lesion caused by the plant-pathogenic rust fungus Hemileia vastatrix (orange) on a leaf of Coffea arabica . b. Atractilina parasitica (orange) on colonies of Meliola clerodendricola (black mildew) on a leaf of Clerodendrum capitatum. c. Niveomyces sp. on Ophiocordyceps dipterigena on a dead fly, collected by Romina Gazis in Florida, USA (photo: Carlos Sendoya Corrales).

2. Relevant terminology

2.1. Hyperparasitism, mycoparasitism, and fungicolous fungi

The term hyperparasitism was introduced by Boosalis (1964) as an alternative for mycoparasitism and used in reference to the phenomenon of one fungus parasitic on another fungus. Although similar, these terms imply two different things. "Mycoparasitism" is a phenomenon in which one fungus (the mycoparasite) parasitizes another fungus (the host), regardless of whether the host is a saprotroph, mutualist, parasite, or commensalist (Karlsson *et al*., 2018; Moore *et al*., 2020). Moreover, mycoparasitism typically involves cell wall degradation and, in most cases, penetration of the host cells, e.g., as in the mycoparasitic activity of *Trichoderma harzianum* against *Rhizoctonia solani* (Altomare *et al*., 1999; Atanasova *et al*., 2013; Sun *et al*., 2019).

In contrast, "hyperparasitism" occurs only if the host is also a parasite (Bermúdez-Cova *et al*., 2022; Faticov *et al*., 2022; Haelewaters *et al*., 2018a; Piepenbring, 2015). It is important to note that hyperparasitic fungi use different methods to interact with their hosts (Boosalis, 1964; Jeffries, 1995), from hyphae or haustoria that penetrate host tissues to hyphal contact without penetration, to buffer cells that may facilitate flow of nutrients from host to parasite (Barnett and Lilly, 1958). For a fungus – or any other organism – to be considered a hyperparasite, it needs to negatively impact host fitness, otherwise it would be referred to

as a "hypermutualist" or "hypercommensal" (Kaishian *et al.*, 2023; Northrup *et al.*, 2021). A hyperparasitic interaction consists of *at least* three trophic levels (**Fig. 2**): a primary host, which is parasitized by a primary parasite, which serves as secondary host to a secondary parasite or hyperparasite.
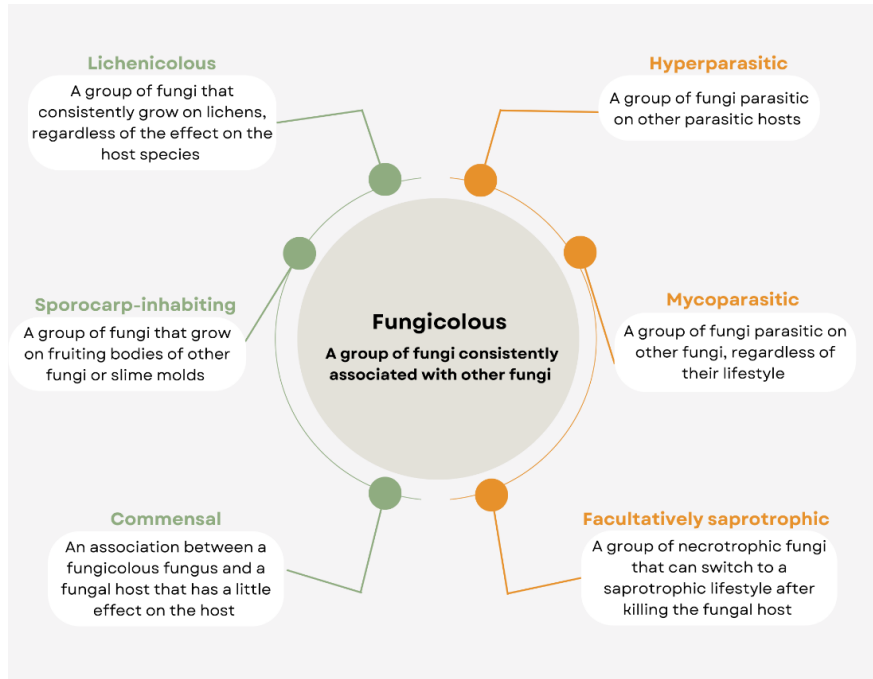

Figure 2: Definitions of important terms, based on Butler (1954), Hawksworth (1981), Jeffries and Young (1994), Alexopoulos (1996), Lawrey and Diederich (2003), Gams et al. (2004), Piepenbring (2015), Haelewaters et al. (2018a), Sun et al. (2019), Moore et al. (2020), Bermúdez-Cova et al. (2022), and Diederich et al. (2022). Definitions colored in green represent fungicolous fungi that cause little or have no effect on the fungal hosts, while definitions colored in orange represent fungi that have negative effects on the hosts.

The general term "fungicolous fungus" refers to a fungus that is consistently associated with other fungi (**Fig. 2**; Gams *et al.*, 2004; Hawksworth, 1981; Sun *et al.*, 2019). Researchers may also refer to fungi as fungicolous when the exact nature of the trophic relationship is not known (Barnett, 1963; Barnett and Binder, 1973). A distinction between hyperparasites, mycoparasites, and fungicolous fungi is made in the literature for several reasons. First, hyperparasitic fungi are frequently studied for their potential use in biocontrol of economically important parasites and pathogens (Brotman *et al.*, 2010). Second, they represent an opportunity to study trophic cascades and natural dynamics of predation in both host and parasite populations (**Fig. 3**; Parratt and Laine, 2016). Finally, parasitism of another organism that is strongly or obligately reliant on a specific host, has potential impacts on the dispersal and evolution of that organism, which parasites of non-pathogens may not experience.
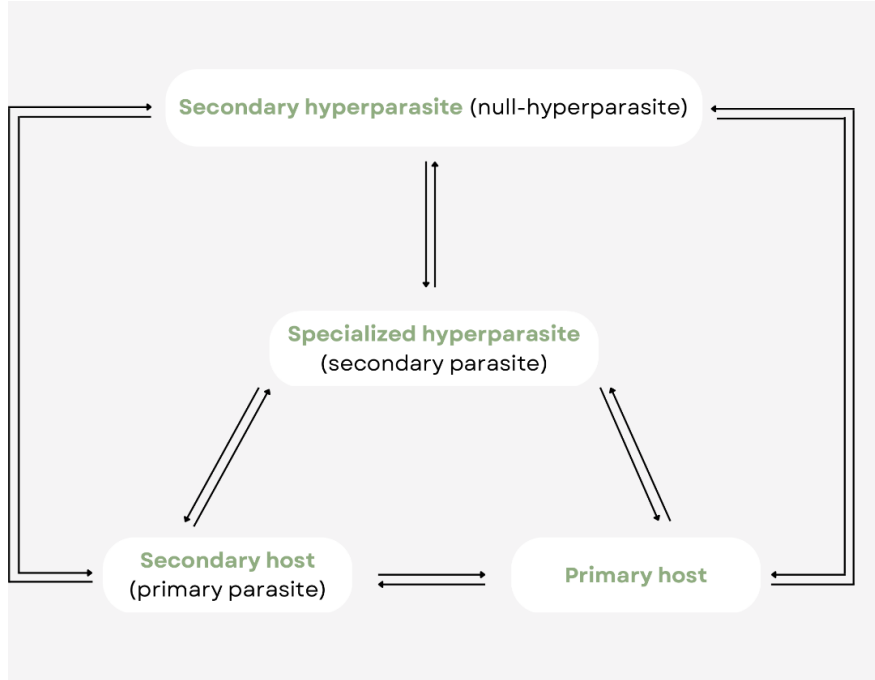
Figure 3: Multitrophic interactions between primary hosts, secondary hosts, and their specialized primary and secondary hyperparasites.

Some host species of hyperparasites do not necessarily have a fixed ecological strategy but rather exist on an ecological continuum during their life cycle, e.g., ranging from parasitism to saprotrophism. This can be illustrated by *Armillaria* spp., which are necrotrophs on various tree species. Once the host tree has died, *Armillaria* switches to a saprotrophic strategy, decaying the same tree substrate. *Armillaria* species themselves have been recorded as hosts for at least two agaricioid mycoparasites, namely *Collybia cookei* and *Entoloma abortivum* (see below). Nomenclatural issues may also arise when hyperparasites have multiple host species, some of which are parasites themselves whereas others may be saprotrophs. A prime example of this are species of *Trichoderma* , which infect both pathogenic and saprotrophic hosts (Jeffries and Young, 1994). In such cases use of the term "hyperparasite" maybe situational, depending on the ecological context of the host. Therefore, a "one-definition-fits-all" approach is unlikely to encapsulate the diversity of interactions observed in nature.

2.2. Null-hyperparasitism

It may happen that a hyperparasite is attacked by another parasite (Gállego Berenguer, 2007). Borkar (2020) refers to these secondary hyperparasites as null-hyperparasites, as they "nullify" the biocontrol activity of the primary hyperparasite. In a recent *in-vivo* experiment, this author showed that strains of *Aspergillus niger* and *Bacillus thermophilus* have the ability to parasitize the fungus *Trichoderma hamatum*, a common hyperparasite of the groundnut pathogen, *Sclerotium rolfsii*.

3. Types of hyperparasitic relationships

One of the ways fungal hyperparasites are defined is based on the state (living or dead) of the primary parasite (Barnett and Binder, 1973). Fungi that exploit living host tissue or cytoplasm are considered biotrophs,

4

whereas necrotrophs kill host cells and then utilize host biomass (Benjamin *et al.*, 2004; Jeffries and Young, 1994). Biotrophic hyperparasites typically have a narrower range of hosts and develop specialized structures to interact with their hosts (Jeffries, 1985, 1995). Examples of biotrophic hyperparasites and their parasitic hosts are given in **Table 1**. Many of these biotrophic hyperparasites form haustoria or specialized hyphal branches involved in absorption of food from host mycelia or sclerotia (Kirk *et al.*, 2008). Necrotrophs frequently use antifungal compounds in so-called hyphal interference (when the host is a fungus) or destroy the cell wall and membranes of host tissue to gain access to cellular contents, or use a combination of these strategies (Jeffries and Young, 1994).

Hyper-parasite	Primary parasite	Primary host	Evidence	Reference(s)
Agaricomycetes				
Collybia cookei	Armillaria spp.	Plant	Growth on host	Ludwig, 2012
Entoloma abortivum	Armillaria spp.	Plant	Carpophoroid morphology	Lindner *et al.*, 2001
Tremellomycetes				
Filobasidium elegans	Alternaria spp.	Plant	Growth on host	Bandoni *et al.*, 1991
Filobasidium floriforme	Alternaria spp.	Plant	Haustoria observed in co-culture	Bandoni *et al.*, 1991
Filobasidium globisporum	Pleospora spp.	Plant	Growth on host	Bandoni *et al.*, 1991
Heteromycophaga tremellicola	Tremella philippinensis	Plant	Growth in host hymenium	Roberts and Spooner, 1998
Phragmoxenidium mycophilum	Rhizoctonia fusispora	Fungus	Haustoria observed - TEM	Oberwinkler *et al.*, 1990
Sigmogloea tremelloidea	Coniochaeta spp.	Plant	Haustoria observed - growth on host	Bandoni and Krug, 2000
Sirotrema parvula	Lophodermium pinastri	Plant	Haustoria observed - growth on host	Bandoni, 1986
Sirotrema pusilla	Hypoderma spp.	Plant	Haustoria observed - growth on host	Bandoni, 1986
Sirotrema translucens	Lophodermium spp., *Hypodermella* spp.	Plant	Haustoria observed - growth on host	Bandoni, 1986
Tetragoniomyces uliginosus	Rhizoctonia sp.	Plant	Haustoria observed - TEM	Oberwinkler and Bandoni, 1981
Tremella bryonectriae	Bryonectria cuneifera	Plant	Growth on host	Döbbeler, 2019
Tremella colpomaticola	Colpoma quercinum	Plant	Haustoria observed - direct interaction not observed	Hauerslev, 1999
Tremella karstenii	Colpoma juniperi	Plant	Haustoria observed - direct interaction not observed	Hauerslev, 1999

6

Mycoparasitic hyperparasites can also be classified based on the part of the host that is infected. For example, many species appear to attack only sclerotia (e.g., *Tyrannicordyceps fratricida*; Kepler *et al.*, 2012), spores (*Olpidium uredinis*; Berndt, 2013), or entire sporocarps (e.g., *Polycephalomyces* spp.; Kepler *et al .*, 2013).

Fungi categorized as hyperparasites include many mycoparasites, but as mentioned above, other fungi have non-fungal parasites as hosts. These include many animals such as insects and nematodes that are further discussed below. It is likely that there are important physiological and chemical differences among hyperparasites whose hosts belong to different kingdoms of life, and this is yet another way that hyperparasites can be categorized.

4. Diversity of hyperparasitic fungi

Hyperparasitic fungi are found across the fungal tree of life (**Fig. 4**), from Cryptomycota to former 'zygomycetes' to Basidiomycota (Gleason *et al.*, 2012; Jeffries, 1985; Lutz *et al.*, 2004) (**Fig. 5**). The genus *Trichoderma* (Sordariomycetes: Hypocreales) includes the best studied mycoparasites, some of which are hyperparasites of plant pathogens (Brotman *et al.*, 2010; Elad *et al.*, 1980). Hypocreales is an order with 320 genera that are rich in hyperparasites of fungi parasitic on plants, animals, and other fungi (Sung *et al.*, 2007; Wijayawardene *et al.*, 2022) (**Fig. 6**). *Akanthomyces lecanii* is a member of this order that exploits hosts in two different kingdoms: the coffee rust fungus, *Hemileia vastatrix* (Pucciniomycetes: Pucciniales), and the coffee scale insect, *Coccus viridis* (Vandermeer *et al.*, 2009). Having hosts that themselves are obligate associates with coffee plants as parasites, potentially enables this dynamic hyperparasite to maintain various reservoirs for dispersal through time and physical space in the environment (Jackson *et al.*, 2016). Many other prominent and well-studied groups of hyperparasites are representatives of Dothideomycetes. Some examples of hyperparasites in Dothideomycetes are *Ampelomyces* spp. (Pleosporales) on powdery mildews (Kiss *et al.*, 2004) and *Cladosporium* spp. (Capnodiales) on various parasitic hosts (Moricca *et al.*, 2005).
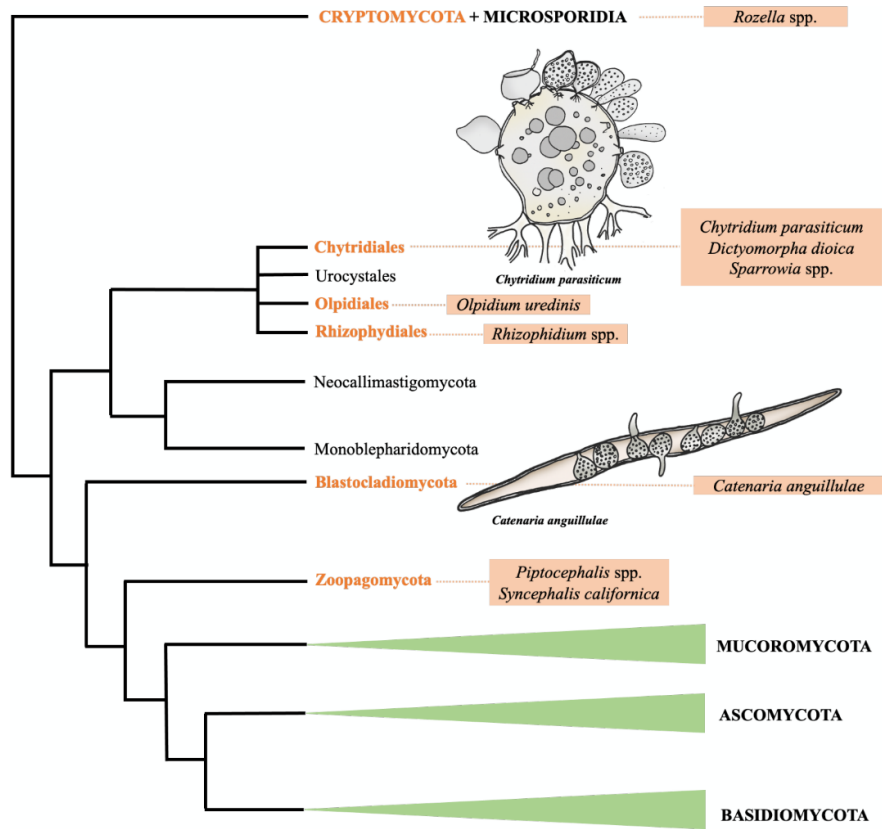
Figure 4: A simplified phylogeny of the Kingdom Fungi. Taxonomic groups in which hyperparasites are known are indicated in orange. Examples of hyperparasitic fungi are shown in orange boxes. Phylogenetic hypothesis taken and modified from Kendrick (2017), Spatafora et al. (2017), and Amses et al. (2022).
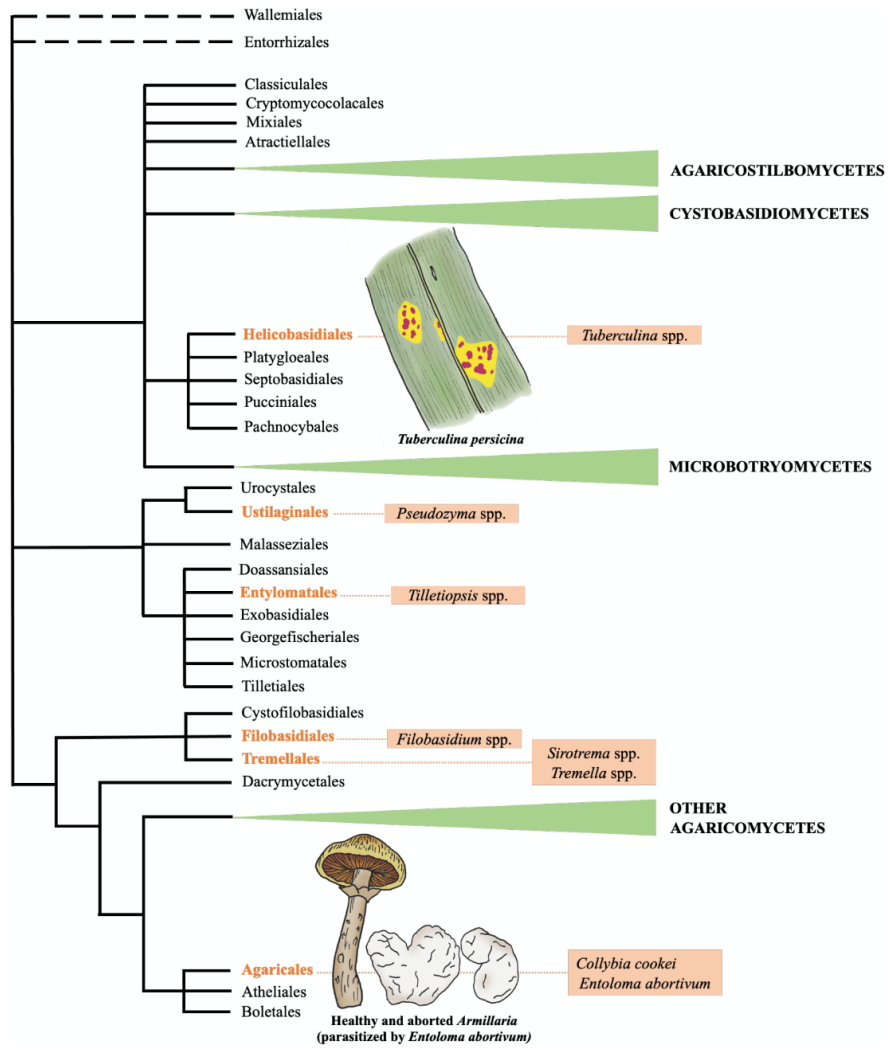
Figure 5: Simplified phylogenetic hypothesis of Basidiomycota. Taxonomic groups in which hyperparasites are known are indicated in orange. Examples of hyperparasitic fungi are shown in orange boxes.
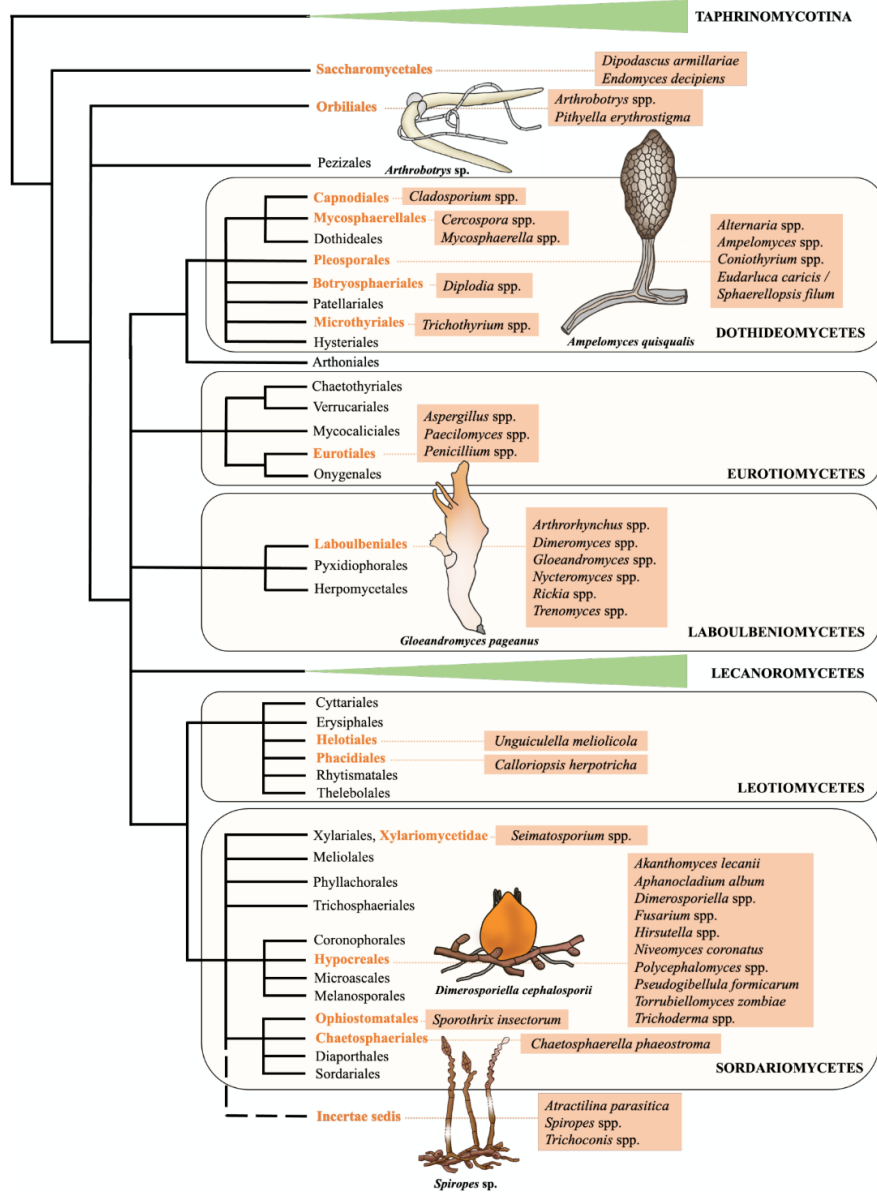
Figure 6: Simplified phylogenetic hypothesis of Ascomycota. Taxonomic groups in which hyperparasites are known are indicated in orange. Examples of hyperparasitic fungi are shown in orange boxes.

Fungal hyperparasites can also infect non-fungal hosts. The most common examples are nematophagous fungi able to parasitize plant-parasitic nematodes (Zhang *et al.*, 2020). Other than the egg stage, nematodes are capable of moving through their environments, posing a challenge to immobile and relatively slow-growing fungal parasites. However, some parasitic fungi have evolved to infect mobile stages of nematodes by means of specialized predation structures such as trapping structures to immobilize nematodes (Jiang *et al.*, 2017; Zhang *et al.*, 2020). Many lineages of fungi are known to trap or prey on parasitic nematodes, such as species of *Arthrobotrys*, *Monacrosporium* (Orbiliomycetes: Orbiliales), *Drechmeria*, *Fusarium*, *Harposporium*, *Hirsutella* (Sordariomycetes: Hypocreales), *Nematophthora* (Oomycota *incertae sedis*), *Paecilomyces* (Eurotiomycetes: Eurotiales), *Pochonia* (Sordariomycetes: Hypocreales), *Verticillium* (Sordariomycetes: Glomerellales), among others (Siddiqui and Mahmood, 1996).

4.1. Hyperparasites of plant-parasitic microfungi

Plant-parasitic microfungi are frequently colonized by hyperparasitic fungi that are able to penetrate the hyphae, the spores, and/or the reproductive structures of their fungal hosts (Gams *et al.*, 2004; Lumsden, 1992; Zhan *et al.*, 2014). Some of these parasites are specific to certain groups of plant pathogens and have garnered interest as biocontrol agents, such as *Ampelomyces quisqualis*(Dothideomycetes: Pleosporales) (**Fig. 7**), a naturally occurring hyperparasite of powdery mildews (Faticov *et al.*, 2022; Huth *et al.*, 2021). The most common plant-parasitic hosts include species of powdery mildews (Erysiphaceae), black mildews (Meliolales), tropical tarspot fungi (Phyllachorales), rusts (Pucciniales), and smuts (Ustilaginales and further orders) (Gams *et al.*, 2004; Hawksworth, 1981). Information about hyperparasitic fungi on plant-parasitic microfungi is scattered through literature, and there is no detailed treatment of biotrophic plant pathogens and their hyperparasites, as most publications deal with individual groups of fungi (Bermúdez-Cova *et al.*, 2022). Therefore, the following sections offer a summary of hyperparasites attacking these major groups of plant pathogens.
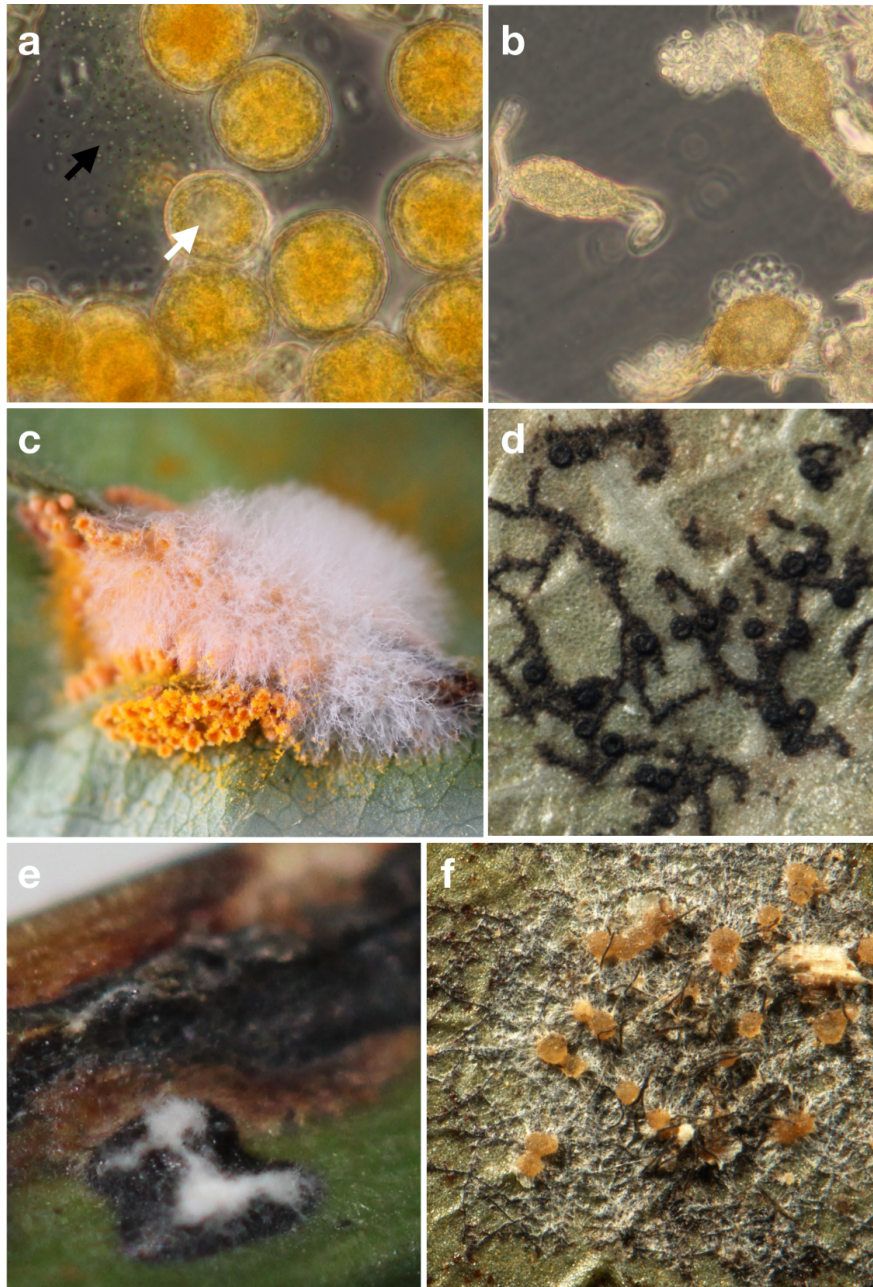
Figure 7: Hyperparasitic fungi on plant-associated microfungi. a. Zoospores (black arrow) and zoosporangium (white arrow) of Olpidium uredinis inside a urediniospore of a rust fungus (Pucciniales). b. Picnidia of Ampelomyces quisqualis. c. Fusarium sp. on aecidia of Puccinia coronata on a leaf of Rhamnus cathartica. d. Trichothyrium sp. on a colony of Meliola sp. e. White hyphomycete on pycnidia of Camarotella costaricensis (Sordariomycetes: Phyllachorales) on a leaf of Acrocomia aculeata. f. Paranectriella sp. on a colony of Meliola sp.

12

4.1.1. Hyperparasites of powdery mildews

Species of Erysiphaceae (Leotiomycetes: Helotiales; Haelewaters *et al.*, 2021b), the powdery mildews, are frequently attacked by species of hyperparasitic fungi belonging to the genus *Ampelomyces* , such as the type species *A. quisqualis* (Faticov *et al.*, 2022; Huth *et al.*, 2021; Parratt and Laine, 2016; Tillenaere *et al.*, 2014). This is a destructive, obligate, intracellular parasite that occurs on both the sexual and asexual stages of Erysiphaceae (Hawksworth, 1981). *Ampelomyces quisqualis* is able to form pycnidia inside the fungal host perithecia and/or hyphae, resulting on the reduction or complete halt of sexual and asexual sporulation of the powdery mildew species (Hawksworth, 1981; Legler *et al.*, 2016). Powdery mildew colonies infected by *Ampelomyces* spp. are easily identified by a change in color, from white to brown (Faticov *et al.*, 2022; Németh *et al.*, 2019). While molecular studies have revealed that *Ampelomyces* may comprise at least four to seven species, the taxonomy within the genus is unresolved (Németh *et al.*, 2019, 2021).

There are other less common species of fungi reported to be growing on colonies of Erysiphaceae, such as the hyphomycetes *Acremonium byssoides*, *Akanthomyces lecanii*, and *Aphanocladium album* (Sordariomycetes: Hypocreales) (Hawksworth, 1981). The usually saprotrophic fungus *Cladosporium oxysporum* (Dothideomycetes: Capnodiales) was found to arrest the development and maturation of the ascospores of *Phyllactinia corylea* (Rao and Pavgi, 1978). Species of *Pseudozyma* (Ustilaginomycetes: Ustilaginales) and *Tilletiopsis* (Exobasidiomycetes: Entylomatales) are occasionally found parasitizing powdery mildews (Gafni *et al.*, 2015; Gams *et al.*, 2004; Klecan *et al.*, 1990).

4.1.2. Hyperparasites of black mildews

An approximate 200 species of fungi are reported to be hyperparasitic on colonies of black mildews (Sordariomycetes: Meliolales). They include organisms from diverse systematic groups, and therefore comprise species producing a high diversity of reproductive structures, such as synnemata, pycnidia, apothecia, perithecia, and catathecia, among others (Bermúdez-Cova *et al.*, 2022). The most common hyperparasites of black mildews are species of the genera *Atractilina*, *Spiropes* (Pezizomycotina *incertae sedis*), *Dimerosporiella* (Sordariomycetes: Hypocreales), and *Trichothyrium* (Dothideomycetes: Microthyriales) (Bermúdez-Cova *et al.*, 2022; Deighton and Pirozynski, 1972; Ellis, 1968; Pirozynski, 1977; Rossman, 1987; Rossman *et al.*, 1999). Hyperparasites of Meliolales are contact-biotrophic fungi and prevent their host from producing spores and ascomata (Stevens, 1918; Toro, 1952). The current systematic position of almost all species of hyperparasitic fungi of Meliolales is unknown due to two reasons: the description of many of these predated the molecular era and technical problems make DNA extractions and PCR amplifications challenging (see **7. Molecular studies of hyperparasitic fungi**).

4.1.3. Hyperparasites of tropical tar spot fungi

Tropical tar spot fungi (Sordariomycetes: Phyllachorales), along with Erysiphaceae and Meliolales, are among the most frequently hyperparasitized fungal lineages (Cannon, 1991; Hawksworth, 1981). Parbery (1978) listed some common hyperparasitic fungi of *Phyllachora* and *Linochora* species, namely *Phaeodothis winteri* (Dothideomycetes: Pleosporales), as well as species of *Cercospora*, *Mycosphaerella* (Dothideomycetes: Mycosphaerellales), *Seimatosporium* (Sordariomycetes: Amphisphaeriales), and other dematiaceous fungi. Other potential hyperparasites of Phyllachorales are cited by Baker and Dale (1951), Sivanesan and Kranz (1975), and Sutton (1980). Caution is warranted when interpreting fungal associates of tar spot fungi. The anamorph–teleomorph connections in Phyllachorales are not well understood; asexual states may be misinterpreted as hyperparasites, and vice versa (M. Mardones, personal communication). Moreover, it may be difficult to determine whether an associated fungus is a hyperparasite of the tar spot fungus or simply uses the cavities or the lesions as entrance for direct plant parasitism (Hawksworth, 1981).

Hyperparasitic fungi of Phyllachorales use different strategies to infect their hosts. Some hyperparasites grow through the perithecial ostiole of the tar spot fungus to expose their conidiophores, whereas others form a narrow layer of conidiogenous cells closely adjacent to the inner perithecial layer of the phyllachoralean fungus and remain almost invisible on the leaf surface (M. Mardones, personal communication). The coelomycete

13

Diplodia sp. (Dothideomycetes: Botryosphaeriales), for example, forms its pycnidia inside the ascomata of *Phyllachora sacchari* (Rao, 1967). Hyperparasitized colonies of *Phyllachora* can be recognized by their dull surface and the necrotized host tissue around them (Gams *et al.*, 2004).

4.1.4. Hyperparasites of rusts

More than 80 species and approximately 30 genera of fungi can parasitize rust fungi (Pucciniomycetes: Pucciniales) and are, mostly asexual forms of Ascomycota (Gams *et al.*, 2004; Hawksworth, 1981; Kranz, 1981; Leinhos and Buchenauer, 1992; Zhan *et al.*, 2014; Zheng *et al.*, 2017). In the genus *Cladosporium*, *C. aecidiicola*, *C. cladosporioides*, *C. pseudocladosporioides*, *C. sphaerospermum*, *C. tenuissimum,* and *C. uredinicola* have been reported as hyperparasites of rust fungi (Keener, 1954; Mendgen, 1981; Moricca *et al.*, 1999; Sharma and Heather, 1978; Srivastava *et al.*, 1985; Sun *et al.*, 2019; Torres *et al.*, 2017; Traquair *et al.*, 1984; Tsuneda and Hiratsuka, 1979; Vandermeer *et al.*, 2009; Wang *et al.*, 2016; Zhan *et al.*, 2014). *Cladosporium* species are in close contact with the cells of the rust fungus, through formation of appressoria and penetration of the host cells by mechanical force or through the production of lytic enzymes (Assante *et al.*, 2004; Moricca *et al.*, 2001; Nasini *et al.*, 2004).

Species of the genus *Tuberculina* (Pucciniomycetes: Helicobasidiales) are known only to be parasitic on rust fungi (in their asexual stage), living in association with more than 150 host species from at least 15 genera (Hawksworth, 1981; Lutz *et al.*, 2004). The most common species are *T. maxima* and *T. persicina*, reported from species of *Cronartium* and *Gymnosporangium*, respectively (Hawksworth, 1981; Hubert, 1935). *Tuberculina* species have an alternating life cycle (Lutz *et al.*, 2004) with morphologically and ecologically distinct sexual and asexual stages, which were formerly classified into different genera: *Helicobasidium* for the sexual stage and *Tuberculina* for the asexual stage.

In their asexual stage, *Tuberculina* species produce lilac to violet sporodochia-like structures growing on the sori of rust fungi. Cytoplasmic contacts between host and parasite are facilitated by micrometer–fusion pores, structures that are unique among Basidiomycota (Bauer *et al.*, 2004). In the sexual stage, these species are phytopathogens that form purplish crust-like sporocarps on living and dead plant material, causing violet root rot on a multitude of plant host species.

Quasiramularia phakopsoricola (Ustilaginomycetes, Basidiomycota) is a mycoparasite on the rust *Phakopsora ampelopsidis* and represents the only known mycoparasitic member among Ustilaginomycotina (Kolařík *et al.*, 2021). This hyperparasite resembles the hyphomycetous morphology of *Ramularia* species (Dothideomycetes, Ascomycota), and its affinity to Basidiomycota was only proven by phylogenetic analyses. Sexual reproduction in this species is not known, and the host-parasite interaction mechanism remains to be investigated.

The most common hyperparasite of rust fungi is the pycnidial fungus *Sphaerellopsis filum* (Dothideomycetes: Pleosporales). This is a biotrophic hyperparasite that grows mostly in the uredinia of its host (Gams *et al.*, 2004; Keener, 1934). Through the production of enzymes, it is able to penetrate urediniospores to inhibit their germination (Carling *et al.*, 1976; Leinhos and Buchenauer, 1992; Stähle and Kranz, 1984). *Sphaerellopsis filum* has a broad host range among rust fungi; and has been documented from over 360 species in 30 genera (Leinhos and Buchenauer, 1992).

Akanthomyces lecanii and *Aphanocladium album* (Sordariomycetes: Hypocreales) are necrotrophic hyperparasites that penetrate and destroy spores of *Puccinia graminis* (Gams *et al.*, 2004; Leinhos and Buchenauer, 1992). The infection of urediniospores by *A. lecanii* induces precocious teliospore formation, which may be a self-defense mechanism of the rust fungus against the hyperparasite (Koç and Défago, 1983). Species of *Acremonium*, *Fusarium, Simplicillium* (Sordariomycetes: Hypocreales), *Alternaria* (Dothideomycetes: Pleosporales), and *Verticillium* have also been reported as hyperparasites (Buchenauer and Leinhos, 1982; Gams, 1975; Wollenweber, 1934; Zheng *et al.*, 2017). Many other potential parasites of rust fungi are cited by Hawksworth (1981), Gowdu and Balasubramanian (1988), Leinhos and Buchenauer (1992), and Gams *et al.* (2004).

4.1.5. Hyperparasites of smuts and bunts

Reports of hyperparasitic fungi on smut and bunt fungi (Basidiomycota: Ustilaginomycotina) are scarce (Hawksworth, 1981). Species of *Fusarium* may grow on *Ustilago* spp., and the infections by these parasites can render the edible galls produced by *Ustilago maydis* poisonous (Gams *et al.*, 2004; Wollenweber and Reinking, 1935). *Aphanocladium album*, a common parasite of rust fungi, has also been reported growing on teliospores of Ustilaginales (Koç and Défago, 1983). Species of *Tilletiopsis* (Exobasidiomycetes: Entylomatales) have been found growing on lesions caused by *Entyloma* (Exobasidiomycetes: Entylomatales), although their hyperparasitic activity has not been demonstrated (Brady, 1960).

4.2. Zoosporic hyperparasites

Zoosporic hyperparasites have been reported among Fungi in Blastocladiomycota, Chytridiomycota, and Cryptomycota, and among zoosporic fungus-like protists such as Hyphochytriomycota, Labyrinthulomycota, and Oomycota (Gleason *et al.*, 2014), all three of which are now recognized as belonging to the Stramenopila lineage of Eukaryotes (Keeling and Burki, 2019; Wijayawardene *et al.*, 2022). Zoosporic parasites can grow as epibionts on the surface of their hosts by means of specialized structures such as rhizoids, or as endobionts (i.e., intracellularly) being completely submerged within their hosts (Held, 1973, 1974; Gleason *et al.*, 2012; Karling, 1960). There is a third type of association, such as in hyphal-forming zoosporic organisms, where interactions between hyphae of the hyperparasite and the primary parasite can be observed (Gleason *et al.*, 2014). This is the case, for example, for the interactions of the oomycete *Pythium oligandrum* and hyphae of its plant-parasitic oomycete hosts, *Pythium* spp. and *Phytophthora* spp. (Benhamou *et al.*, 1999).

Some parasites have evolved to grow on closely related host taxa. These parasites are known as "adelphoparasites" (Goff and Zuccarello, 1994). Species of *Pythium* are often parasitized by species of the same genus, such as *P. acanthium*, *P. mycoparasiticum*, *P. nunn*, *P. oligandrum*, and *P. periplocum* (Berry *et al.*, 1993; Deacon, 1976; Deacon and Henry, 1978; Lutchmeah and Cooke, 1984; Martin and Hancock, 1987; Vesely, 1977). It is also common among chytrids to be parasitized by other chytrids. Species of the same genus may be both parasite and host and, in some cases, individuals of the same species parasitize each other (Frenken *et al.*, 2017; Karling, 1960). For example, *Chytridium parasiticum* is a hyperparasite of *Chytridium suburceolatum*, which is itself a parasite on *Rhizidium richmondense* (Gleason *et al.*, 2014; Willoughby, 1956). Adelphoparasitism is a common phenomenon among zoosporic hyperparasites, but it is also known in other taxa, such as in *Tyrannicordyceps* and *Claviceps* species (Kepler *et al.*, 2012).

The most comprehensive taxonomic treatments on zoosporic hyperparasites were done by Karling (1942a, 1942b) and Sparrow (1960). More studies, however, are necessary to describe both the diversity of these organisms and their interactions.

4.3. Sordariomycetes hyperparasites of zombie-ant fungi

The genus *Ophiocordyceps* contains species of insect pathogens and mycoparasites (**Fig. 8**), a few of which are famous because of their ability to manipulate the behavior of their insect hosts (Eberhard *et al.*, 2014; Roy *et al.*, 2006). Species of the *Ophiocordyceps unilateralis* clade induce climbing and biting behaviors in ant hosts of the tribe Camponotini (Evans *et al.*, 2011). This is known as "summit disease", which is common to many arthropod parasites across multiple lineages of the fungal kingdom (Evans, 1989; Marikovsky, 1962; Roy *et al.*, 2006). These behavioral manipulations increase transmission chances of *Ophiocordyceps* fungi and have earned them the moniker "zombie-ant fungi". These pathogens are not immune to becoming parasitized themselves. While formal descriptions of hyperparasites of zombie-ant fungi are few and scattered, the presence of hyperparasites has certainly been noted by mycologists who study *Ophiocordyceps* across the globe (Andersen *et al.*, 2012; Araújo *et al.*, 2020, 2022; Mongkolsamrit *et al.*, 2021).
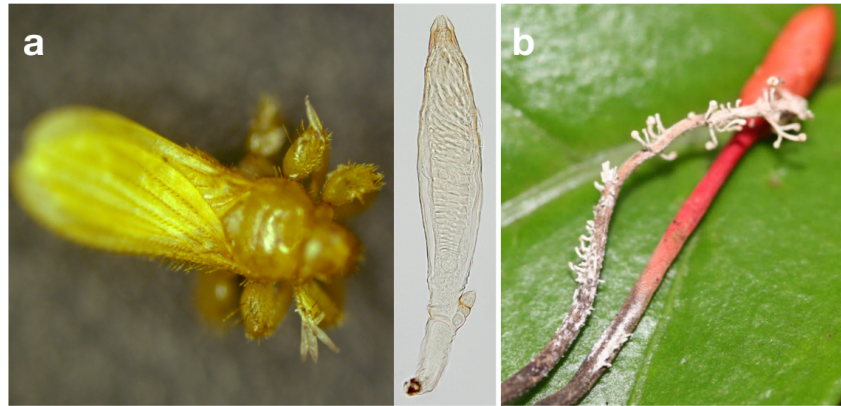
Figure 8: Hyperparasitic fungi on insect hosts. a. Nycteromyces streblidinus on the legs of a Trichobius joblingi bat fly (left) collected from a Carollia perspicillata bat, and a female thallus of the Laboulbeniales microfungus at higher magnification (right). b. Polycephalomyces cf. yunnanensis (Hypocreales: Ophiocordycipitaceae) parasitizing Ophiocordyceps nutans (Ophiocordycipitaceae), a pathogen of stink bugs (Hemiptera: Pentatomidae). In the background, a bright pink uninfected ascoma of O. nutans is shown for contrast.

Multiple hyperparasite species can be associated with a single *Ophiocordyceps*–ant species pair. Three species – *Pseudogibellula formicarum*, *Torrubiella carnata/liberiana/pseudogibellulae* (Hypocreales), and *Sporothrix insectorum* (Ophiostomatales) – were found on *Ophiocordyceps paltothyrei*, which infects *Palthothyreus tarsatus* ants in Ghana (Araújo *et al.*, 2020). Additionally, two recently described hyperparasite species, *Niveomyces coronatus* and *Torrubiellomyces zombiae*, are associated with *Ophiocordyceps camponoti-floridani*, infecting and manipulating the carpenter ant *Camponotus floridanus* in Florida, USA (Araújo *et al.*, 2022). This suggests that future work on the hyperparasites of zombie-ant fungi will likely reveal a wealth of undescribed species that could be mined for their abilities to affect animal-infecting fungi.

Beyond documenting their diversity, the extent to which hyperparasites affect the life span and transmission of their *Ophiocordyceps* hosts, as well as their molecular and cellular mechanisms remain to be investigated. A field study on *O. camponoti-floridani* suggests that both hyperparasites associated with this ant parasite co-occur in the same wilderness areas and harshly limit its transmission chances; when hyperparasitized, 10–40% of ant cadavers were observed with perithecia of *O. camponoti–floridani*, while this percentage was significantly higher (76%) in non-parasitized *Ophiocordyceps* (Will *et al.*, 2022). However, only 4% of *Ophiocordyceps*-manipulated ant cadavers had visible hyperparasite growth within the 1-year timespan of the study. Moreover, while new *T. zombiae* infections were found year-round, *N. coronatus* appeared to have a more seasonal occurrence (Will *et al.*, 2022). The disease dynamics of hyperparasites associated with *Ophiocordyceps* spp. might add a species-specific layer of complexity to the understanding of these multitrophic interactions.

4.4. Laboulbeniales hyperparasites

Laboulbeniales are an order of enigmatic microfungi that form three-dimensional multicellular thalli instead of hyphae and are associated with a living host for the entire duration of their life cycle (Haelewaters *et al.*, 2012). Hosts include a variety of Arthropods: harvestmen and mites (subphylum Chelicerata: class Arachnida); millipedes (Myriapoda: Diplopoda); and numerous insect lineages (Hexapoda: Insecta), such as ants, beetles, cockroaches and termites, crickets, earwigs, flies, lice, thrips, and true bugs. Some of the

arthropod hosts of Laboulbeniales are parasites themselves, which results in hyperparasitic associations. The study system that is researched most in depth is that of Laboulbeniales associated with bat flies (Diptera: Nycteribiidae and Streblidae), which are bloodsucking ectoparasites of bats (Mammalia: Chiroptera).

Bat fly-associated Laboulbeniales were discovered in the 1850s, although at that time known as acanthocephalan worms (Kolenati, 1857). By 1932, the year that marks the death of Roland Thaxter who described hundreds of species of Laboulbeniales, five species of Laboulbeniales from bat flies were described. Twenty years later, Merola (1952) described a sixth species, and it took another 65 years for any taxonomic contributions in this system (Haelewaters *et al.*, 2017b). To date, 18 species in four genera are known to parasitize bat flies (Haelewaters *et al.*, 2021a; Liu *et al.*, 2020; Van Caenegem *et al.*, 2023; W. Van Caenegem and D. Haelewaters, unpublished data): four species of *Arthrorhynchus* , two species of *Dimeromyces*, ten species of *Gloeandromyces*, and two species of *Nycteromyces* (**Fig. 8**). In addition, Haelewaters *et al* . (2020) revealed that *Arthrorhynchus eucampsipodae* is a complex of at least two species segregated by host genus. Given that *A. eucampsipodae* has been reported on flies in four genera (de Groot *et al.*, 2020), it could very well be a complex of four species, possibly more.

Some of the Laboulbeniales species associated with bat flies penetrate their hosts with haustoria, rhizoidal structures that make contact with the body cavity for nutrition and as a holdfast. Haustorial Laboulbeniales are those that have recently been referred to as the true biotrophic members of the order (Reboleira *et al.*, 2021). Bat flies with haustorial Laboulbeniales are often deformed and their integument is severely blackened (due to melanization) at the site of infection (Jensen *et al.*, 2019). The extent of damage to the hosts is largely unknown and probably varies among Laboulbeniales (Kaishian *et al.*, 2023). However, Szentiványi *et al.* (2020) showed that *Arthrorhynchus* spp. reduced bat fly survival in *Penicillidia conspicua* bat flies.

Different studies point at very low parasite prevalences of bat flies with Laboulbeniales, ranging from 2.2% to 9.0% (Blackwell, 1980; Haelewaters *et al.*, 2017a, 2018b; Szentiványi *et al.*, 2018; Walker *et al.*, 2018). Except some regional studies focusing on prevalence of parasites and one study reviewing tritrophic associations globally and analyzing host specificity patterns (de Groot *et al.*, 2020), other aspects remain unstudied. Efforts are being made towards a global tritrophic traits database to study some of these aspects based on records resulting from standardized fieldwork (Haelewaters *et al.*, 2021a). One question of interest is how environmental pressures such as changing landscapes and warming climate affect parasitism at these multiple levels.

Other examples of Laboulbeniales that have parasites as hosts are found in two other genera: *Rickia* on mites of ants, *Salganea* cockroaches, *Nasutitermes* termites, and beetles in different families; and *Trenomyces* on lice of birds, cows, foxes, and rats as well as on louse flies of primates (*Lepilemur* sp.). In addition, species of *Dimeromyces* are not only associated with bat flies, they are also found on mites of beetles in different families. It should be mentioned that it is not always clear whether these mite hosts are truly parasites or rather commensals in relation to the primary host. Finally, written notes by Jean Balazuc at the Muséum National d'Histoire Naturelle, Paris reveal an unpublished genus of Laboulbeniales from a human ectoparasite (the sucking louse *Pediculus humanus*, order Psocodea).

4.5. Basidiomycetous hyperparasites

Examples of basidiomycetous hyperparasites are surprisingly scarce. Roughly 200 species of mycoparasites have been described in this phylum, with a dozen of them being putative hyperparasites. Examples of hyperparasitism have been documented in four classes: Agaricomycetes, Tremellomycetes (Agaricomycotina), Ustilaginomycetes (Ustilaginomycotina), and Pucciniomycetes (Pucciniomycotina). The best studied group of hyperparasites within Basidiomycota is Helicobasidiales (Pucciniomycetes). This order comprises species of *Tuberculina* , which are hyperparasites of rusts (**4.1.4. Hyperparasites of rusts**). Within Agaricomycetes and Tremellomycetes, evident examples of hyperparasitism are extremely rare, but see **Table 1** for specific examples.

In Agaricomycetes, only two examples of hyperparasitism are known. Both *Collybia cookei* and *Entoloma*

abortivum have been reported as hyperparasites on species of *Armillaria* (Lindner *et al.*, 2001) (**Table 1**). *Armillaria* species are devastating, necrotrophic phytoparasites on various tree species, but may shift to saprotrophism once the host tree has died. Most species within Tremellomycetes are mycoparasites and lichen parasites (Diederich *et al.*, 2022; Millanes *et al.*, 2011; Weiss *et al.*, 2014). However, host species identity is often uncertain (only identified to genus or form group) or not known at all. This makes it very hard to estimate which proportion of these mycoparasites are to be considered hyperparasites. Further, for the majority of these hyperparasites, no cultures nor genetic data are available, and their classification remains tentative based on (micro)morphological similarities (Schoutteten *et al.*, 2023; Weiss *et al.*, 2014).

5. Ecological role of fungal hyperparasitism

Although a common phenomenon in nature, the real impacts of hyperparasitism on the ecology and evolution of the organisms involved and its cascading effects throughout food webs is understudied. In the broad sense, hyperparasites are analogous to predators, where the secondary hosts (primary parasites) act as herbivores and the primary hosts replace primary producers. Therefore, as predators, hyperparasites are able to shape ecosystem stability through top–down cascades (Parratt and Laine, 2016). Hyperparasitic fungi also influence the dynamics of the interactions between the primary hosts and the primary parasites, increase the complexity of the food webs, and play a significant role in regulating population sizes of either partner (Gleason *et al.*, 2014; Sandhu *et al.*, 2021). By decreasing the fitness of their host, hyperparasites may essentially exert a net positive effect on the fitness of the primary host (Northrup *et al.*, 2021; Sandhu *et al* ., 2021). However, a convincing conceptual framework is lacking, and tractable model systems to study hyperparasitic interactions in natural populations are scarce (Péter *et al.*, 2022; Parratt and Laine, 2016).

It is hypothesized that zoosporic parasites have a role in the structure and function of aquatic food webs, by lengthening food chains and carbon paths. As their life cycles are shorter, zoosporic hyperparasites also increase and accelerate the energy flow among trophic levels, by producing biomass in the form of zoospores and zoosporangia that enter the food web contributing different types of energy for predators (Gleason *et al.*, 2014).

The range of interactions among hyperparasites, their hosts (i.e., the secondary hosts), and the primary hosts is wide and complex, and sometimes difficult to establish (Gleason *et al.*, 2014; Kiss, 2001). Studies on host specificity in hyperparasitic fungal systems are scarce (but see Barnett and Lilly, 1958; Jeffries and Young, 1978), and those examining all three trophic levels in the same analysis are even rarer. One recent study analyzed the ecological interactions among the three levels of the multitrophic network among bats, bat flies, and microfungi and found that bat flies are much more host specific at the community-level compared to their Laboulbeniales hyperparasitic fungi (de Groot *et al.*, 2020).

6. Evidence of hyperparasitic interactions

Studies of hyperparasitic interactions between fungi and their hosts have been observed both in the field and by microscopy (Kim and Vujanovic, 2018; Moore *et al.*, 2020; Smith *et al.*, 2008). However, in most cases, the antagonistic activity of the hyperparasite is not evident in the field, and the exact interactions may only be revealed under laboratory conditions, when the cultivation of the hyperparasite is possible or when infected primary and/or secondary hosts can be reared.

The associations of hyperparasites and their hosts can be visualized by molecular techniques that employ expression of fluorescent proteins (Hasan *et al.*, 2022). For example, the gene-encoding green fluorescent protein (GFP) was expressed in *Trichoderma* species, which helped to elucidate their interactions with *Pythium ultimum*, the invasion of the hyphae and sclerotia of *Rhizoctonia solani*, and the penetration of the plant-parasitic nematode *Globodera pallida* (Contina *et al.*, 2017; Lu *et al.*, 2004; Sarrocco *et al.*, 2006). Also, Németh *et al.* (2019) used a GFP marker to visualize the life history strategy of *Ampelomyces quisqualis*.

18

Hyperparasitic interactions may be assumed if the parasite causes distinctive morphological or physiological alterations of the primary parasite, with the latter showing signs of phenotypic changes, such as deformation of cells, growth impairment, and changes in color (Gams *et al.*, 2004; Jeffries, 1995; Zheng *et al.*, 2017). For example, urediniospores of *Puccinia striiformis* f. sp. *tritici* collapse and lose viability after being colonized by hyphae of *Alternaria alternata* and *Cladosporium cladosporioides* (Zhan *et al.*, 2014; Zheng *et al.*, 2017). Parasitism may also be assumed when parasites affect the reproductive rate of the hosts, e.g., by decreasing levels of sporulation of fungal hosts. This has been observed for hyperparasites of black mildews, powdery mildews, and rusts and smuts (Bermúdez-Cova *et al.*, 2022; Legler *et al.*, 2016; Zhan *et al.*, 2014; Zheng *et al.*, 2017). The incapability of fungi growing on parasites to be cultured on axenic media, i.e., without their hosts, also serves as an indication that they are obligate hyperparasites (Jeffries, 1995).

7. Molecular studies of hyperparasitic fungi

Hyperparasitic fungi belong to different phylogenetic lineages and have different morphologies, and as a result, no specific set of molecular methods has been developed to study hyperparasites. Yet, despite these differences, researchers frequently encounter similar problems when studying them. Some hyperparasites are minute in size and require non-standard micromanipulation techniques. In addition, many have melanin in their cell walls, which provides rigidity but inhibits PCR amplification and the ability to get high quality DNA (Bermúdez-Cova *et al.*, 2022; Eckhart *et al.*, 2000; Haelewaters *et al.*, 2015).

Because they are part of multitrophic networks, it is common to find hyperparasites intermingled with tissue of the primary parasite and other organisms present in a given sample. This makes the isolation of DNA exclusively from the hyperparasite difficult. Moreover, many hyperparasitic fungi are biotrophs and cannot be grown axenically. The hosts themselves may also be biotrophic, further complicating DNA isolation from either partner. These factors have contributed to a lack of reference sequences for taxonomic and systematics research and also have ramifications even for genomics research; for mycoparasitic hyperparasites, *in silico* attempts at de-novo genome sequencing derived from metagenomic data can be unfeasible because the methods used for separation of host and hyperparasite sequences cannot easily discriminate between the two fungi (Quandt *et al.*, 2017).

Due to the challenges described above, publicly accessible databases are notably lacking in their representation of hyperparasites. As an example, in the latest version of the UNITE database (version 9.0, 27 October 2022) (Nilsson *et al.*, 2018), out of almost 8.4 million ITS sequences, there are only 35 of Laboulbeniales—a taxon with over 2,300 described species and many more yet to be described (Haelewaters *et al.*, 2021a). Not all species in this order are hyperparasites, but many of them are, and as UNITE is the primary database used in environmental microbiome studies (Tedersoo *et al.*, 2022), the paucity of taxa that are represented leads to an underreporting of their presence in nature and therefore our understanding of the natural world.

Generalizations about the genetic "toolkit" that hyperparasitic fungi use are difficult if not impossible to make, due to the phylogenetic and morphological diversity of both the primary parasite and the primary host. However, the nature of individual hyperparasitic relationships can and should be investigated. In one such example, Koch and Herr (2021) used transcriptomics (RNA-seq) to examine the differential expression of genes in both the hyperparasite, *Entoloma abortivum*, and its host, a plant-pathogenic *Armillaria*, during their parasitic interaction compared to expression in their respective sporocarps. Transcripts obtained from the interaction interface are mainly from *E. abortivum*, the hyperparasite, and contain genes hypothesized to be involved in mediating recognition of *Armillaria* and detoxification of compounds produced by the pathogen. Modern techniques such as these now allow for examining the nature of the interaction between the hyperparasite, its primary parasite, and the primary host.

19

8. Hyperparasitic fungi and biological control

Environmental and health concerns caused by the use of chemicals such as fungicides, nematicides, and pesticides have increased the need for alternative measures for the control of pathogens (Moosavi and Zare, 2020; Thambugala *et al.*, 2020). Hyperparasitic fungi play a significant role in controlling pathogens, and they have been used as biological control agents for at least 70 years (Heydari and Pessarakli, 2010; Thambugala *et al.*, 2020). Biocontrol agents represent an alternative to fungicides in disease control (Köhl *et al.*, 2020). The use and utility of biocontrol agents, however, has had limited success (Savita and Sharma, 2019) and more work is needed to fully examine the most appropriate and beneficial applications of specific hyperparasites in biocontrol.

The fungi best studied for their use in biocontrol are species of the genus *Trichoderma* (Brotman *et al.*, 2010; Harman *et al.*, 2004; Motlagh and Samimi, 2013; Reino *et al.*, 2008). Around 90% of fungal biocontrol agents belong to different strains of *Trichoderma*, and currently more than 60% of the effective bio-fungicides are obtained from species of this genus (Abbey *et al.*, 2019; Hermosa *et al.*, 2012). Moosavi and Zare (2020) stated that 25 species of *Trichoderma* have the potential of controlling more than 100 fungal pathogens worldwide. Out of these species, *Trichoderma harzianum* may be considered the most common and commercially developed biocontrol agent used for a wide range of plant-pathogenic fungi. *Trichoderma* species have an antagonistic behavior against bacteria, nematodes, and fungi by inhibiting growth and they may indirectly improve the growth and stress tolerance of the primary plant host (Kumar, 2013; Zhang *et al.*, 2017).

Clonostachys rosea is a hyperparasitic fungus capable of invading various plant-pathogenic fungi, including *Botrytis cinerea, Fusarium* spp., *Rhizoctonia solani,* and *Sclerotinia sclerotiorum* (Barnett and Lilly, 1962; Cota *et al.*, 2008; Jensen *et al.*, 2000; Luongo *et al.*, 2005; Rodríguez *et al.*, 2011), with *C. rosea* strain 67-1 being highly efficient for biocontrol (Zhang *et al.*, 2007; Ma *et al.*, 2011; Sun *et al.*, 2018). Hasan *et al.* (2022) showed that the GFP-marked *C. rosea* strain 67-1 exerts antagonistic activities against *B. cinerea* both *in vitro* and on tomato leaves. The hyperparasite is able to penetrate its host, absorb its nutrients, and eventually disintegrate all of its cells.

Ampelomyces quisqualis has been the subject of numerous investigations on biological control of powdery mildews for over 50 years and, along with species of *Trichoderma*, they are the most common biocontrol agents that have reached international markets (Falk *et al.*, 1995a, 1995b; Kiss *et al.*, 2004). Several cross-inoculation experiments, both *in vitro* and in the field (Angeli *et al.*, 2012; Kiss *et al.*, 2011; Legler *et al.*, 2016; Liang *et al.*, 2007; Németh *et al.*, 2021), have shown that species of *Ampelomyces* are not strictly host specific. This has allowed for biocontrol agents composed of a single strain to be applied to a wide range of powdery mildew species (Németh *et al.*, 2021).

A large number of crop plants are infected by parasitic nematodes (Savita and Sharma, 2019). They represent a major threat to crops worldwide, and due to the toxicity of nematicides, new control strategies against nematodes need to be developed (Poveda *et al.*, 2020). Fungi have shown great potential as nematicidal biocontrol agents (Siddiqui and Mahmood, 1996). Important fungi used in biocontrol of nematodes are *Pochonia chlamydosporia* (Sordariomycetes: Hypocreales), *Purpureocillium lilacinum* (Sordariomycetes: Hypocreales), and *Hyalorbilia oviparasitica* (Orbiliomycetes: Orbiliales) (Lysek and Sterba, 1991). Species of *Trichoderma* are also currently being studied as biocontrol agents of parasitic nematodes.

The processes of commercialization and application of fungi as biocontrol of pests have been slow. This is mainly due to diverse fungal performances under variable environmental conditions in the field as well as their host specificity (Thambugala *et al.*, 2020). The development of new formulations of biocontrol fungi with higher degrees of stability and survival is necessary to overcome this problem (Heydari and Pessarakli, 2010). Commercialization of biological control agents is expensive and involves many steps such as isolation in pure culture, the development of a suitable formulation, mass production, testing efficacy of the product, environmental safety matter assessment, among others (Janisiewicz and Korsten, 2002; Montesinos, 2003).

Moreover, the cultivation of hyperparasites is not always possible and therefore the development of biocontrol products from these fungi remains challenging.

9. Future avenues of research

One of the challenges to studying hyperparasitic fungi includes the ability to recognize the morphology and natural history of both the primary host and the primary parasite in their uninfected states. Currently few experts are trained to identify all of the partners in the different trophic levels of hyperparasitic interactions, which explains the paucity of published literature on this topic. While these hyperparasitic fungal systems are potentially diverse, they are largely unexplored. Multitrophic, multiyear, multisite sampling efforts have been proposed to strengthen future analyses on host specificity patterns and community ecology (Cazabonne *et al.*, 2022; de Groot *et al.*, 2020; Haelewaters *et al.*, 2021a).

In addition to the lack of sampling, little attention has been given to the theoretical framework for systems involving hyperparasites (Sandhu *et al.*, 2021). Most of this work has focused on the use of hyperparasitic fungi in biocontrol experiments, directed toward reducing the damage caused by primary parasites (Day, 2002; Rosenheim *et al.*, 1995). It is essential to understand how parasites interact with their own parasites to effectively control infectious diseases (Parratt *et al.*, 2017).

While much is left unknown about hyperparasitic fungi, the presence and expression of secondary metabolite gene clusters (Quandt *et al.*, 2016, 2018) and their antifungal activities (Wang *et al.*, 2016) among many lineages of mycoparasites including hyperparasites are well documented. The advent of genomics has proven that many species and strains have the ability to produce countless compounds whose activities have the potential for myriad biotechnological and pharmaceutical uses (Keller, 2019). Hyperparasites, many mentioned here in this chapter, likely harbor antifungal compounds that have yet to be discovered and described (Kim *et al.*, 2002; Wicklow *et al.*, 1998). Without more work examining hyperparasitic fungi, these compounds and their potential uses will remain unknown.

Acknowledgments

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PUBLICATION 3

Bermúdez-Cova MA, Krauß A, Sanjur A *et al.* 2023b. Diversity of hyperparasitic fungi on Meliolales (Sordariomycetes, Ascomycota): new species, records, and molecular data from Benin and Panama. Mycological Progress 22, 65. https://doi.org/10.1007/s11557-023-01913-5.

Anlage 3

Erklärung zu den Autorenanteilen an der Publikation (Titel):

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Was hat der Promovierende bzw. was haben die Koautoren beigetragen?

(11)   zu Entwicklung und Planung

Promovierender MABC: 90 %

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(12)   zur Durchführung der einzelnen Untersuchungen und Experimente

Promovierender MABC: 100 %

MABC carried out 100 % of the DNA extraction, amplification (PCR) and sequencing.

(13)   zur Erstellung der Datensammlung und Abbildungen

Promovierender MABC: 55 %

Koautor AK: 10 %

Koautor AS: 5 %

Koautor AT: 10 %

Koautor TAH: 5 %

Koautor NSY: 5 %

Koautor MP: 10 %

MABC collected most of the samples in Panama and Benin in 2020 and 2022, respectively and prepared the figures plates. AK and AT collected some further specimens in Benin in September 2022. MP collected one specimen (MP 53256) in 2016 and collaborated with host plant identification. AS, TAH and NSY collaborated with host plant identification. AS, AT, TAH and NSY collaborated with logistics and sampling in Panama and Benin.

(14)   zur Analyse und Interpretation der Daten

Promovierender MABC: 60 %

Koautor AK: 5 %

Koautor AS: 5 %

Koautor AT: 5 %

Koautor TAH: 5 %

Koautor NSY: 5 %

Koautor MP: 15 %

MABC performed DNA sequence analyses, multiple sequence alignments and phylogenetic reconstructions. Interpretation of the phylogenies from a systematic point of view was done by MABC in collaboration with AK, AS, TAH, NSY and MP. Nomenclatural decisions were taken by MABC and MP.

(15)   zum Verfassen des Manuskripts

Promovierender MABC: 70 %

Koautor AK: 4 %

Koautor AS: 4 %

Koautor AT: 4 %

Koautor TAH: 4 %

Koautor NSY: 4 %

Koautor MP: 10 %

Zustimmende Bestätigungen der oben genannten Angaben:

Datum/Ort   Unterschrift Promovend

Datum/Ort   Unterschrift Betreuer

Datum/Ort   Ggfs. Unterschrift *corresponding author*

DGfM

ORIGINAL ARTICLE

Diversity of hyperparasitic fungi on *Meliolales* (*Sordariomycetes*, *Ascomycota*): new species, records, and molecular data from Benin and Panama

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Abstract

Meliolales (black mildews) is an order of plant parasitic ascomycetous fungi in the tropics and subtropics. They are frequently overgrown and parasitized by other fungi, known as hyperparasites. During the last few years, species of hyperparasitic fungi on *Meliolales* have been collected in Benin and Panama. A new species of *Paranectria* and seven new reports of hyperparasites of different systematic groups are presented here with detailed descriptions and illustrations, together with new data concerning fungal hosts and host plants. The new species is called *Paranectria longiappendiculata*, characterized by exceptionally long appendages carried by the ascospores. New records for Benin and Panama are *Calloriopsis herpotricha*, *Dimerosporiella cephalosporii*, *Isthmospora glabra*, *Isthmospora trichophila*, *Malacaria meliolicola*, *Paranectriella hemileiae*, and *Paranectriella minuta*. *Calloriopsis herpotricha* is recorded for Africa and *D. cephalosporii* and *P. hemileiae* for America for the first time, suggesting an apparently pantropical distribution. Findings show a blatant lack of investigation on hyperparasitic fungi in the tropics. The phylogenetic positions of three of these newly reported species, *C. herpotricha*, *D. cephalosporii*, and *P. minuta*, are shown based on the analysis of internal transcribed spacer (ITS), large subunit (LSU), and small subunit (SSU) rDNA sequences. These sequences were generated in the context of the present study for the first time.

Keywords Black mildews · Hyperparasites · 1 new taxon · ITS/LSU/SSU rDNA · Pantropical distribution

Introduction

Meliolales (*Sordariomycetes*, *Ascomycota*), commonly known as "black mildews", form a large order of biotrophic, obligate plant parasitic fungi in the tropics and subtropics. Species of

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this order develop on leaves, petioles, twigs, and sometimes fruits of vascular plants (Piepenbring et al. 2011; Hongsanan et al. 2015; Zeng et al. 2017). Black mildews cause a reduction of chlorophyll, starch, sugar, proteins, and amino acids in the plant tissues they infect, as well as alterations in the photosynthetic and respiratory rates (Old et al. 2003).

Meliolales are frequently infected by hyperparasites (Hawksworth 1981; Gams et al. 2004). There are approximately 200 species of fungi reported to be hyperparasitic on colonies of *Meliolales* (Bermúdez-Cova et al. 2023), but we expect a much greater number of species to exist in the tropics. Fungal hyperparasites belong to diverse taxonomic groups, and therefore comprise species producing a high diversity of reproductive structures, such as apothecia, catathecia, perithecia, pycnidia, and synnemata, among others. They are generalists concerning genera of *Meliolales*, but many of these hyperparasites seem to be restricted only to meliolalean hosts (Bermúdez-Cova et al. 2022).

Knowledge of species diversity of black mildews in the tropics is still limited. Only three species are known for Benin and 105 for Panama (Piepenbring et al. 2011; Piepenbring et al.

2020; Hofmann and Piepenbring 2021). Information on hyperparasitic fungi of *Meliolales* in Benin and Panama is inexistent (Bermúdez-Cova et al. 2022). For a better understanding of the diversity, evolution, and biology of hyperparasitic fungi, it is necessary to increase sampling efforts and to undertake further morphological, molecular, and ecological studies.

Materials and methods

Sample collection and morphological characterization

Samples of leaves infected with black mildews were opportunistically collected in Western Panama from January to March 2020 and in Benin in February as well as September 2022. For the present study, colonies of *Meliolales* parasitized by hyperparasites were considered. Infected leaves were dried in a plant press and deposited in the herbarium at the Universidad Autónoma de Chiriquí (UCH, specimens from Panama) and in the mycological herbarium of the University of Parakou (UNIPAR) in Benin. If a given sample was large enough, a duplicate was deposited in the Botanische Staatssammlung München (M).

Dried specimens were observed by stereomicroscopy and by light microscopy. Measurements of at least 20 ascospores, conidia, and other structures have been made for each specimen at a magnification of ×600 and ×1000. Measurements are presented as mean value ± standard deviation with extreme values in parentheses. Line drawings were made freehand on scaled paper. Images and drawings were edited with Photoshop (Adobe, San Jose, California).

Host plant identification

Host plants were identified by morphological characteristics and in some cases by molecular sequence data. Morphological identifications were made by comparison with herbarium specimens, literature (e.g., Akoègninou et al. 2006; Condit et al. 2011), and with the help of local botanists. Molecular sequence data for species identifications were obtained by polymerase chain reaction (PCR) for the amplification of the partial region of chloroplast rbcL with the primer pairs rbcLa-F (Levin et al. 2003) and rbcLa-R (Kress et al. 2009). DNA was extracted from approx. 0.05 g of leaf tissue dried with silica gel using the innuPREP plant DNA kit (Analytik Jena, Germany) and following the manufacturer's instructions. Protocols for PCR were carried out as described by Fazekas et al. (2012).

DNA extraction, PCR amplification, and sequencing of fungal DNA

DNA was isolated from the ascomata of dry specimens using the EZNA forensic DNA extraction kit, following the manufacturer's instructions. To extract total genomic DNA, a small amount of clean ascomata were transferred into a sterile Eppendorf tube with approx. 200 μL of distilled water using sterilized tweezers, and trying to avoid picking cells of any other organism associated with the leaves and the colonies of black mildews. The samples were frozen for 24 h at −20 °C and later homogenized for 10–12 min. using a Retsch mixer mill MM301 with TL buffer and 2.5-mm zirconia beads. Isolated DNA was re-suspended in elution buffer and stored at −20 °C.

Two partial nuclear gene regions (ribosomal loci) were amplified and sequenced: For the large subunit nuclear ribosomal DNA (nrLSU, 28S rDNA), the primers LSU1Fd and LSU3Rd (Crous et al. 2009), NL1 and NL4 (O'Donnell 1993), LR0R (Wagner and Ryvarden 2002), and LR5 (Vilgalys and Hester 1990) were used. For small subunit nuclear ribosomal DNA (nrSSU, 18S rDNA), the primers SSU1Fd and SSU3Rd (Crous et al. 2009) were used. For the internal transcribed spacer region of ribosomal DNA (ITS), the primers ITS5 and ITS4 (White et al. 1990) were used. The PCR mixtures consisted of 1 μL genomic DNA, $15 \times MgCl_2$ reaction buffer (Bioline, Luckenwalde, Germany), 25 mM $MgCl_2$, 25 μM of each dNTP, 10 μM of each primer, and 5 U Taq DNA polymerase (VWR) in a total volume of 30 μL. Cycling parameters of the PCR for ITS, LSU, and SSU were as follows: initial denaturation at 94 °C for 3 min, followed by 35 cycles of amplification [denaturation at 94 °C for 30 s, primer annealing at 52 °C for 30 s and primer extension at 72 °C for 45 s], and a final extension at 72 °C for 5 min, followed by storage at 8 °C. PCR products were checked on 1.5% agarose electrophoresis gels containing HDGreenPlus DNA stain. Amplified PCR products were purified with the Cycle Pure Kit (VWR-Omega, USA). Sequencing was performed at Seqlab GmbH, Germany.

Numerous attempts were made to obtain DNA sequence data of ITS, LSU, and SSU regions from all the specimens collected in the context of this study. Except for four specimens, these attempts failed.

Phylogenetic analyses

Consensus sequences of trace files were generated with Geneious 10.2.2 (https://www.geneious.com, Kearse et al. 2012) and searched against GenBank (https://www.ncbi.nlm.nih.gov/, Benson et al. 2014) with MegaBLAST. Ambiguous and miscalled bases were corrected, when possible, after examination of the corresponding chromatogram files. Sequences with a high similarity were aligned with MAFFT v. 7 using the L-INS-i algorithm (Nakamura et al. 2018). The alignments were manually checked by using MEGA v. 7 (Kumar et al. 2016). Gblocks v. 0.91b (Talavera and Castresana 2007) was used to remove poorly aligned positions and divergent regions from the DNA alignment. Phylogenetic analyses of this study were conducted by

applying maximum likelihood (ML) in RAxML-HPC2 v.8.2.12 (Stamatakis 2014) on XSEDE (Miller et al. 2010) and Bayesian phylogenetic inference with the program MrBayes 3.2.6. (Ronquist et al. 2012) on XSEDE (Miller et al. 2010), available on the CIPRES Science Gateway web portal (http://www.phylo.org/sub_sections/portal/). The alignments and trees were deposited in TreeBASE (http://purl.org/phylo/treebase/phylows/study/TB2:S30529).

Results

Apothecioid hyperparasites

Calloriopsis herpotricha (Berk.) R. Sant., *Svensk bot. Tidskr.* 45(1): 300, 1951 (Figs. 1 and 2).

≡ *Peziza herpotricha* Berk., *Hooker's J. Bot. Kew Gard. Misc.* 3: 16, 1851.

≡ *Helotiella herpotricha* (Berk.) Sacc., *Syll. fung.* (Abellini) 8: 477, 1889.

= *Calloria meliolicola* P. Henn., *Botanisch. Jahrb.* 25: 509, 1898.

≡ *Coryne meliolicola* (P. Henn.) v. Höhnel, *Sitzungsber. Kaiserl. Akad. Wiss., Math.-Naturwiss. Kl., Abt.* 1. 118: 106, 1909.

= *Peziza gelatinosa* Ell. & Mart., *Amer. Nat.* 17: 1283, 1883.

≡ *Orbilia gelatinosa* Sacc., *Syll. Fung.* 8: 624, 1889.

≡ *Coryne gelatinosa* (Sacc.) Rehm, *Ann. Mycol.* 5: 518, 1907.

≡ *Calloriopsis gelatinosa* (Sacc.) Sydow, *Ann. Mycol.* 15: 254, 1917.

Colonies composed of white hyphae covering the colonies of *Meliola* sp. Hyphae thin-walled, septate, 2–3 μm, hyaline. Apothecia 400–600 μm diam., disc pale orange to orange when old, margin slightly paler, translucent. Gelatinous material present throughout the hymenium, subhymenium, ectal excipulum and medullary excipulum. The subhymenium is composed of tightly interwoven hyphae. The ectal excipulum is composed of septate parallel hyphae which are swollen at the tips. Asci clavate, thick-walled especially in young asci, 40–52 μm, 8-spored. Paraphyses filamentous, unbranched, 1–3 μm, sometimes swollen at the tip. Ascospores ellipsoid to fusoid, sometimes curved, (10–)13–16 × 3–6 μm, mostly 1-septate, hyaline, smooth.

Anamorph – Not known.

Specimens examined – On *Meliola* sp. on living leaves of *Coffea arabica*, Benin, Atlantique, Attogon, Niaouli Forest, 6° 44′ 42″ N 2° 7′ 50″ E, 69 m, 28 February 2022, M.A. Bermúdez, A. Tabé, I. Agonglo, O.P. Agbani, N.S. Yorou, MB178; on *Meliola* sp. on living leaves of *Coffea arabica*,
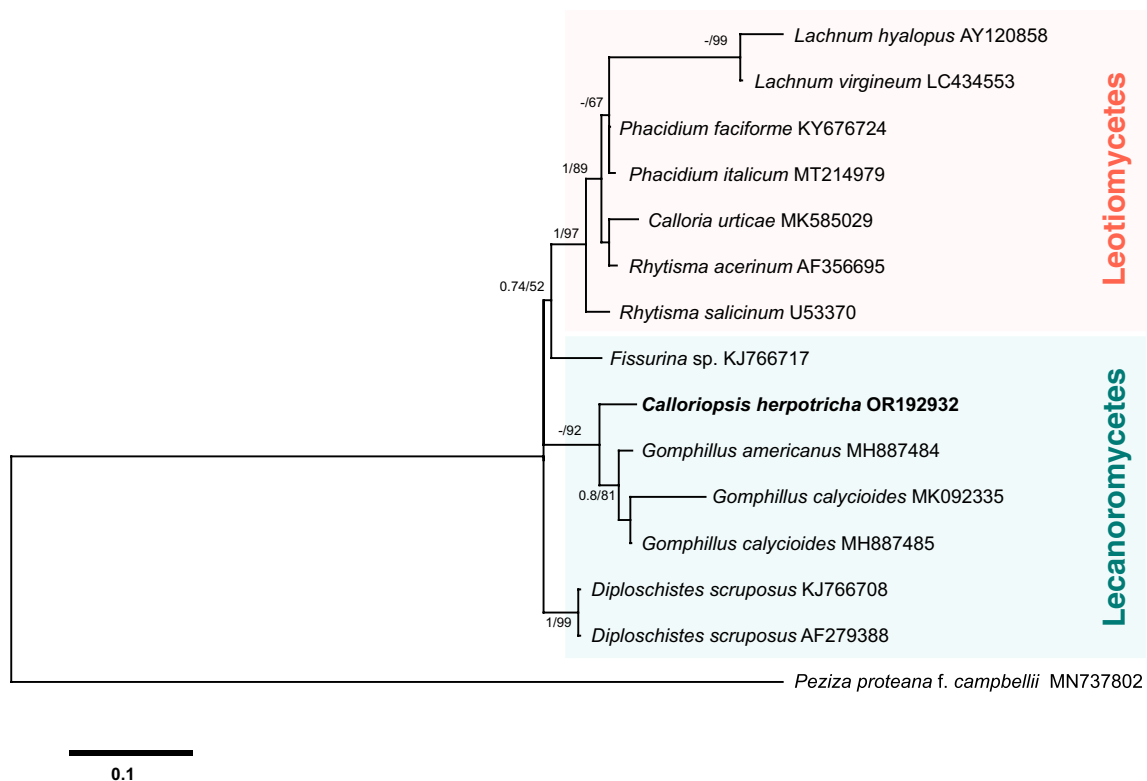

Fig. 1 Phylogenetic tree inferred from the maximum likelihood analysis of SSU sequences of members of the *Leotiomycetes* and *Lecanoromycetes*, including a new sequence for *Calloriopsis herpotricha*. The tree is rooted with *Peziza proteana* f. *campbellii* (*Pezizomycetes*). Bootstrap values and posterior probabilities are indicated above the branches. Sequences downloaded from GenBank are cited with accession numbers

Fig. 2 *Calloriopsis herpotricha* (**AK4H**). **a** Apothecia growing on colonies of *Meliola* sp. on living leaves of *Coffea arabica*; **b** part of a longitudinal section of an apothecium. Dots indicate the presence of gelatinous material; **c** young and mature asci as well as paraphyses; **e** ascospores, shown in optical section (the thickness of the wall is indicated only in the drawing on the left-hand side). Scale bars: 300 μm (**a**); 20 μm (**b**); 6 μm (**c**); 3 μm (**d**)
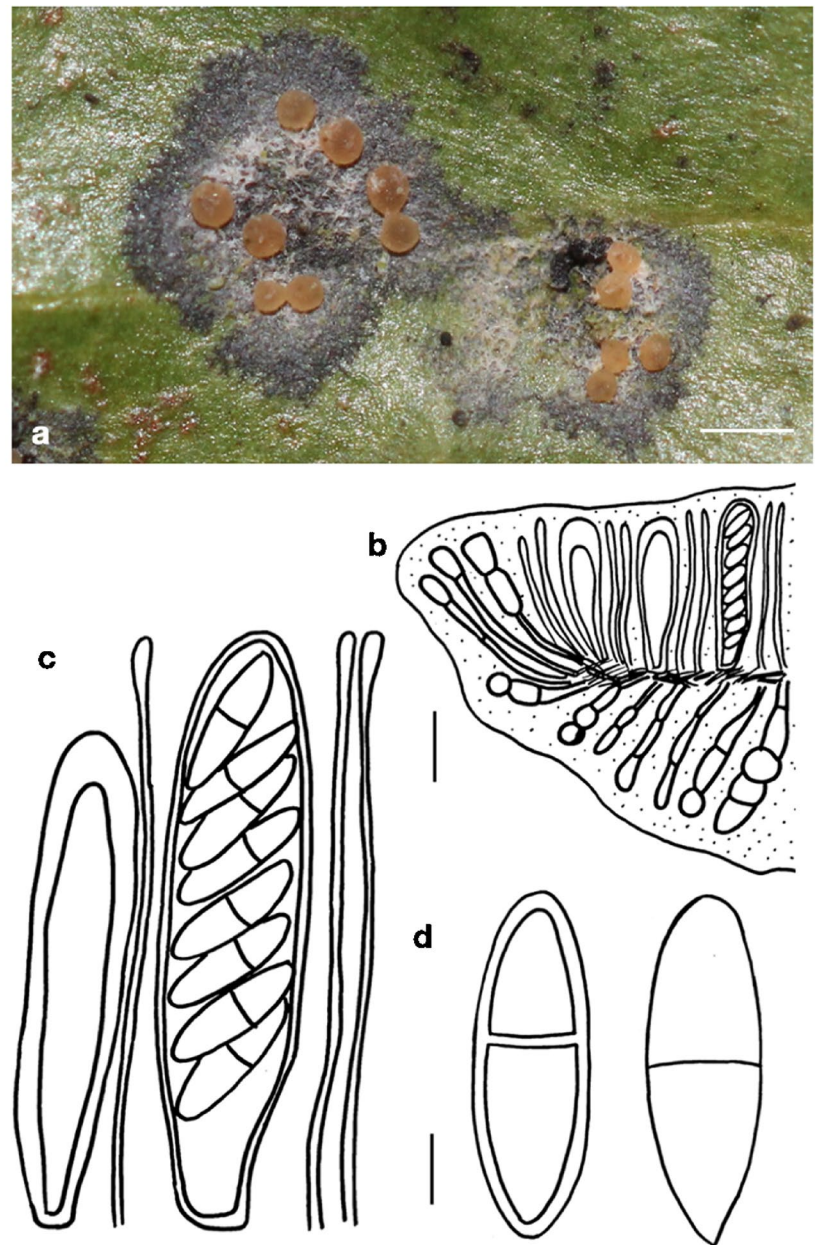
Benin, Atlantique, Attogon, Niaouli Forest, 6° 44′ 23″ N 2° 8′ 26″ E, 119 m a.s.l., 19 September 2022, A. Krauß, A. Tabé, O. Koukol, N.S. Yorou, AK4H (UNIPAR, M, GenBank accession number: OQ800930).

Known hosts and distribution – On *Meliola* sp. on living leaves of *Persea palustris* (*Lauraceae*) in the USA (Ellis and Martin 1883); on living leaves of an unknown plant host in Pará, Brazil (Hooker 1851; Saccardo 1889); on *Meliola* sp. on living leaves of *Phragmites* sp. (*Poaceae*) in Papua New Guinea (Hennings 1898); on *Meliola ramosii* on living leaves of *Homonoia riparia* (*Euphorbiaceae*) in the Philippines; on *Perisporiaceae* on living leaves of *Scaevola* sp. (*Goodeniaceae*) in Hawaii (Cash 1938); on *Meliola* sp. on living leaves of an unknown tree in the Philippines (Santesson 1951); on

Meliola substenospora on living leaves of *Phragmites* sp. (*Poaceae*) in Java, Indonesia (Pfister 1976); on *Meliola* sp. on living leaves of herbs in Puerto Rico (Pfister 1976); without host data in Guadeloupe, France (Pfister 1976); on *Meliola* sp. on living leaves of *Nyssa* sp. (*Nyssaceae*) in the USA (Pfister 1976); on *Meliola* sp. on living leaves of *Coffea arabica* (*Rubiaceae*) in Benin (this study). *C. arabica* is a new host of *C. herpotricha*, and the hyperparasite and the species of *Meliolales* are new records for Benin. This is also the first record of *C. herpotricha* in Africa.

Illustrations – This species was illustrated by Pfister (1976).

Notes – Two species of apothecioid hyperparasitic fungi have been reported to parasitize *Meliolales*, namely, *Calloriopsis herpotricha* and *Unguiculella meliolicola*, both

belonging to the *Leotiomycetes* (*Phacidiales* and *Helotiales*, respectively; Bermúdez-Cova et al. [2022]). The monotypic genus *Calloriopsis* was proposed by Sydow and Sydow ([1917]) and is based on a parasitic discomycete which occurs on *Meliola* and related dark parasites. *Calloriopsis herpotricha* differs from other fungi of the *Leotiomycetes* by the parasitic habit and the gelatinous material that is present in all parts of the apothecium, including the hymenium (Pfister [1976]; Baral and Marson [2001]).

Sequence data – The SSU rDNA sequence obtained from fresh material of *C. herpotricha* (specimen AK4H) is 948 bp long. In the tree inferred from the analysis of SSU sequences of 14 specimens of *Leotiomycetes* and *Lecanoromycetes* (Fig. [1]), the sequence of *C. herpotricha* is located within a strongly supported clade that comprises sequences of species of *Lecanoromycetes* which were obtained from lichenized fungi. Some lineages of non-lichenized *Ascomycota* are known to be derived from lichenized ancestors by the loss of the lichen symbiosis in favor of a saprotrophic, lichenicolous, or parasitic mode of nutrition (Lutzoni et al. [2001]; Hawksworth [2015]; Honegger [2022]). Examples of these include non-lichenized members of *Arthoniales* (*Arthoniomycetes*) and *Ostropales* (*Lecanoromycetes*; Kendrick [2017]). The foregoing and the fact that the sequences of the species of *Calloriopsis* clustered together with other sequences of species of *Ostropales* suggest that the genus *Calloriopsis* may belong to the *Lecanoromycetes* and not to the *Leotiomycetes* as previously assumed. A sequence for *C. herpotricha* is provided here for the first time.

Four sequences of an unidentified species of *Calloriopsis* are available in GenBank (accession numbers: MF322776, MF322774, OM103051, and OQ800930). The specimens that yielded these sequences were found on decayed twigs and branches of *Cornus sanguinea* L. (*Cornaceae*) and *Fraxinus excelsior* L. (*Oleaceae*) in Luxembourg (unpublished data provided by the herbarium LUX). These sequences also fall within the *Lecanoromycetes*. However, these sequences lack the SSU region; thus, it was not possible to compare them with the sequence of *Calloriopsis herpotricha*. We also obtained a DNA sequence of the ITS region of *C. herpotricha* (GenBank accession number: OR243608). This sequence presents 94% identity with the aforementioned sequences of *Calloriopsis* and other members of the *Lecanoromycetes*, confirming the systematic placement of *C. herpotricha* in the *Lecanoromycetes*.

Dematiaceous hyphomycetes

Isthmospora glabra F. Stevens, *Bot. Gaz.* 65(3): 244, 1918 (Fig. [3]a–c).

Hyphae not evident, conidia in small pulverulent brownish heaps scattered over the colonies of *Meliola* sp. Conidiophores not found. Conidia are isthmospores, composed of 11–12

cells. Two pairs of subglobose thick-walled cells, each cell with rounded horns that are directed upwardly and inwardly, 5–6×4–5 μm, brown, smooth. These cells are connected by a central isthmus made of two cells. Connecting cells oblong with wedge-shaped ends, 3–4 μm diam., pale brown, smooth. On each side of the central cells, two to three flask-shaped cells extend upwardly and outwardly into a continuous cylindrical appendage, (15–)20–21(–24)×1–2 μm, hyaline, smooth.

Teleomorph – *Trichothyrium*-like (according to Hughes [1953]).

Specimen examined – On *Appendiculella sororcula* on living leaves of *Calea pittieri*, Panama, Chiriquí Province, David, Dolega district, Los Algarrobos, Majagua river trail, 8° 29′ 28″ N 82° 25′ 59″ W, approx. 150 m a.s.l., 29 December 2016, M. Piepenbring, A. Villarreal, E. Romero, V. Samudio, MP5326 (UCH10000).

Known hosts and distribution – On *Meliola melastomacearum* on living leaves of *Clidemia hirta* (*Melastomataceae*) in Puerto Rico; on *Meliola bicornis* on living leaves of *Meibomia supina* (*Leguminosae*) in Puerto Rico; on *Meliola glabroides* on living leaves of *Nectandra patens* (*Lauraceae*) and *Simarouba tulae* (*Simaroubaceae*) in Puerto Rico; on *Meliola glabra* on living leaves of unknown host in Puerto Rico (Stevens [1918]); on *Appendiculella sororcula* on living leaves of *Calea pittieri* (*Asteraceae*) in Panama (this study). *A. sororcula* and *C. pittieri* are new hosts of *I. glabra*, and the hyperparasite is a new record for Panama.

Illustrations – This species was illustrated by Hughes ([1953]).

Notes – The genus *Isthmospora* (*Microthyriaceae*, *Microthyriales*) was proposed by Stevens ([1918]) and comprises two species of dematiaceous hyphomycetes with dark conidia consisting of two approximately equal halves connected by an isthmus. Both species of the genus, *I. glabra* and *I. spinosa*, are associated with colonies of black mildews (Damon [1953]).

Isthmospora glabra is characterized by the presence of dark smooth central cells with large hyaline appendages (Stevens [1918]; Hughes [1953]). However, according to Damon ([1953]), this species is congeneric with *Spegazzinia chandleri*.

Isthmospora glabra has always been found on the hyphal mat of *Trichothyrium reptans*, a catathecioid hyperparasite of *Meliolales*. Therefore, *I. glabra* is considered to be the anamorphic stage of *T. reptans* (Hughes [1953]). The specimen examined (MP5326) was also found together with a species of *Trichothyrium* (Fig. [3]a, b); however, the size of the ascospores (10–12×4–6 μm) does not match with the size of ascospores of *T. reptans* (15–20×5–6.5 μm; Hughes [1953]). There is no molecular evidence that supports the anamorph-teleomorph connection between these fungi.

Isthmospora trichophila (Atkinson) Damon, *Bull. Torrey bot. Club* 80: 160, 1953 (Fig. [3]d–e).

≡ *Spegazzinia trichophila* G.F. Atk., *Bull. Cornell Univ.* 3(1): 49, 1897.

Fig. 3 *Isthmospora* **spp. on** *Meliolales*. **a–c** (MP5326). **a** Isthmospores of *Isthmospora glabra* growing together with the hyphal mat and catathecia of *Trichothyrium* sp.; **b** asci and ascospores of *Trichothyrium* sp.; **c** isthmospore of *Isthmospora glabra*; **d, e** *Isthmospora trichophila* (AK4H). **d** Isthmospores growing in scattered heaps (see darker spots) on the hyphal mat of *Trichothyrium* sp.; **e** isthmospores drawn at diverse optical levels. Scale bars: 1 mm (**a, d**); 10 μm (**b**); 5 μm (**c**); 7 μm (**e**)
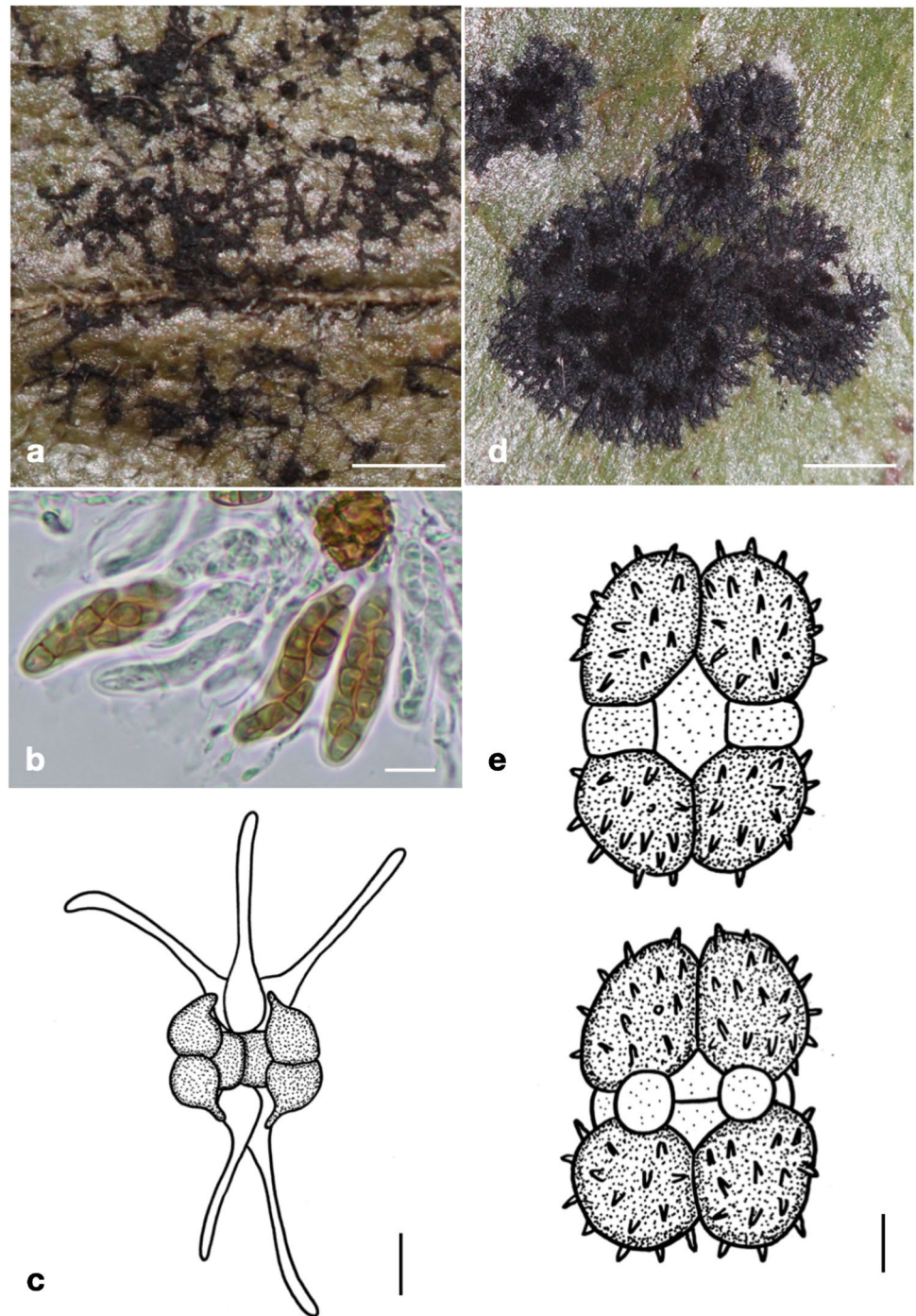
= *Isthmospora spinosa* F. Stevens, *Bot. Gaz.* 65(3): 244, 1918.
= *Spegazzinia coffeae* Henn., apud De Wildeman, *Mission E. Laurent* 3: 318, 1906.
= *Spegazzinia meliolae* Zimm., *Cent. f. Bakt. II*: 8: 221, 1902.
= *Spegazzinia meliolicola* Henn., *Hedwigia* 43: 398, 1904.

Hyphae not evident, conidia in small pulverulent brownish heaps scattered over the colonies of *Meliola* sp. Conidiophores erect, parallel, short, pale brown to brown. Conidia are isthmospores, composed of 11 cells: two pairs of subglobose cells, (14–)16–17(–23) × 11– 14(–19) μm, dark brown, echinulate, with spines up to 2 μm long. The cells are connected by a central isthmus made of three central cells that are oblong with wedge-shaped ends, 3–4 μm wide; there is a single central cell at the upper level and two cells resulting from a septation of another cell at the lower level. Two outer cells more or less oblong, 3–4 μm diam., hyaline, are attached on both sides of the central cells (one cell on each side). At the

base of each outer cell a basal cell, 2–3 μm wide, hyaline, is attached.

Teleomorph – *Trichothyrium*-like (according to Hughes 1953).

Specimens examined – On *Meliola* sp. on living leaves of *Xylopia frutescens*, Panama, Chiriquí Province, Cochea, trail to Cochea river, 8° 32′ 36″ N 82° 23′ 03″ W, 181 m a.s.l., 26 February 2020, M.A. Bermúdez, A. Sanjur, A. Villarreal, MB109 (UCH); on *Meliola* sp. on living leaves of an unknown host plant, Panama, Chiriquí Province, David, Cuesta de Piedra, 8° 41′ 13″ N 82° 36′ 33″ W, 903 m a.s.l., 6 March 2020, M.A. Bermúdez, S. Samaniego, MB118 (UCH); on *Meliola* sp. on living leaves of *Coffea arabica*, Benin, Atlantique, Attogon, Niaouli Forest, 6° 44′ 42″ N 2° 7′ 50″ E, 69 m a.s.l., 28 February 2022, M.A. Bermúdez, A. Tabé, I. Agonglo, O.P. Agbani, N.S. Yorou, MB178; on *Meliola* sp. on living leaves of *Coffea arabica*, Benin, Atlantique, Attogon, Niaouli Forest, 6° 44′ 23″ N 2° 8′ 26″ E, 119 m a.s.l., 19 September 2022, A. Krauß, A. Tabé, N.S. Yorou, O. Koukol, AK4H (UNIPAR, M).

Known hosts and distribution – On *Meliola anacardii* on living leaves of *Anacardium occidentale* (*Anacardiaceae*) in Indonesia; on *Meliola psidii* on living leaves of *Psidium guajava* (*Myrtaceae*) in Brazil (Saccardo and Saccardo 1906); on *Meliola* sp. on living leaves of *Coffea* sp. (*Rubiaceae*) in Ubangi, tropical Africa (Saccardo and Trotter 1913); on *Meliola psidii* on living leaves of *Psidium guajava* (*Myrtaceae*) in Puerto Rico; on *Meliola chiococcae* on living leaves of *Chiococca alba* (*Rubiaceae*) in Puerto Rico; on *Meliola byrsonimae* on living leaves of *Byrsonima lucida* (*Malpighiaceae*) in Puerto Rico; on *Meliola smilacis* on living leaves of *Smilax coriaceae* (*Smilacaceae*) in Puerto Rico; on *Meliola helleri* on living leaves of *Myrcia splendens* (*Myrtaceae*) in Puerto Rico; on *Meliola praetervisa* on living leaves of *Coccolobus sintenisii* and *Coccolobus pyrifolia* (*Polygonaceae*) in Puerto Rico; on *Meliola philodendri* on living leaves of *Philodendron krebsii* (*Araceae*) in Puerto Rico (Stevens 1918); on *Meliola* sp. on living leaves of *Xylopia frutescens* (*Annonaceae*) in Panama (this study); on *Meliola* sp. on leaves of *Coffea arabica* (*Rubiaceae*) in Benin (this study). *Coffea arabica* and *X. frutescens* are new hosts of *I. spinosa*, and the hyperparasite is recorded here for Benin and Panama for the first time.

Illustrations – This species was illustrated by Hughes (1953), Damon (1953) and Tubaki (1963).

Notes – *Isthmospora trichophila* (*Microthyriaceae*, *Microthyriales*) is morphologically similar to species of *Spegazzinia* (*Apiosporaceae*, *Sordariomycetes*), and several known species are easily confused (Damon 1953). However, the complex morphology of the isthmospores and the association with species of *Meliolales* are strong features to distinguish *I. trichophila* from other species of dematiaceous hyphomycetes (Damon 1953; Hughes 1953).

Isthmospora trichophila has always been recorded growing on the hyphal mat of *Trichothyrium asterophorum* (*Microthyriaceae*, *Microthyriales*), a catathecioid hyperparasite of *Meliolales*; thus, it is considered to be the anamorphic stage of *T. asterophorum* (Hughes 1953). The specimens examined were also found together with a species of *Trichothyrium*, which could not be identified because it was not fertile. There is no molecular evidence that supports the anamorph-teleomorph connection between these two species of fungi.

Perithecioid hyperparasites

Dimerosporiella cephalosporii (Hansf.) Rossman & Samuels, *Stud. Mycol.* 42: 23, 1999 (Figs. 4, 5, and 6).

   ≡ *Calonectria cephalosporii* Hansf., *Mycol. Pap.* 15: 117, 1946.
   ≡ *Nectriopsis cephalosporii* (Hansf.) Samuels, *Mem. New York Bot. Gard.* 48: 38, 1988.

Colonies white, cottony, growing on *Meliola* spp. Hyphae septate, 1.7 μm wide, hyaline. Perithecia superficial, globose, (100–)110–150(–220) μm diam., yellow to orange, slightly translucent, not changing color in KOH, smooth; perithecial hairs arising from perithecial apex, septate, unbranched, (10–)17–25(–35) × 3–5.5 μm, wall 0.5–1 μm thick. Perithecial wall 7–9 μm wide, composed of small cells; perithecial apex formed by hyphae that grow outwardly to form perithecial hairs, and inwardly to form periphyses. Asci clavate, apex simple, (25–)32–45(–53) × (6–)7–9(–10) μm, 8-spored. Ascospores completely filling each ascus, ellipsoidal to fusiform, biguttulate, (8.5–)10–15.5(–18) × 1.7–4 μm, 1-septate, hyaline, smooth.

Anamorph – *Acremonium*-like anamorph with conidiophores arising from aerial mycelium, mononematous, macronematous, septate, monophialidic. Phialides thick-walled, with a distinctive collarette, (30–)40–50 μm long × 3–5 μm wide at the base, tapering to 1 μm width at the tip, hyaline. Conidia oblong to ellipsoidal, unicellular, (5–)7.5–9(– 12) × (1.5–)2–3(–3.5) μm, hyaline, smooth.

Specimens examined – On *Meliola* sp. on leaves of *Olyra latifolia*, Panama, Chiriquí Province, David, Botanical Garden of the Universidad Autónoma de Chiriquí (UNACHI), 8° 25′ 55″ N 82° 27′ 4″ W, 34 m a.s.l., 23 January 2020, M.A. Bermúdez, MB86 (UCH13408); on *Meliola* sp. on leaves of *Olyra latifolia*, Panama, Chiriquí Province, David, Los Algarrobos, Majagua river trail, 8° 28′ 47″ N 82° 24′ 46″ W, 80 m a.s.l., 26 February 2020, M.A. Bermúdez, MB113 (UCH13407); on *Meliola pinnatae* on leaves of *Paullinia pinnata*, Benin, Atlantique, Allada, Sékou, 6° 38′ 59″ N 2° 11′ 46″ E, 48 m a.s.l., 15 February 2022, M.A. Bermúdez, A. Tabé, D. Dongnima, M. Piepenbring, N.S. Yorou, MB139 (UNIPAR, M, GenBank accession number: OQ787065); on *Meliola pinnatae* on leaves of *Paullinia pinnata*, Benin, Atlantique,
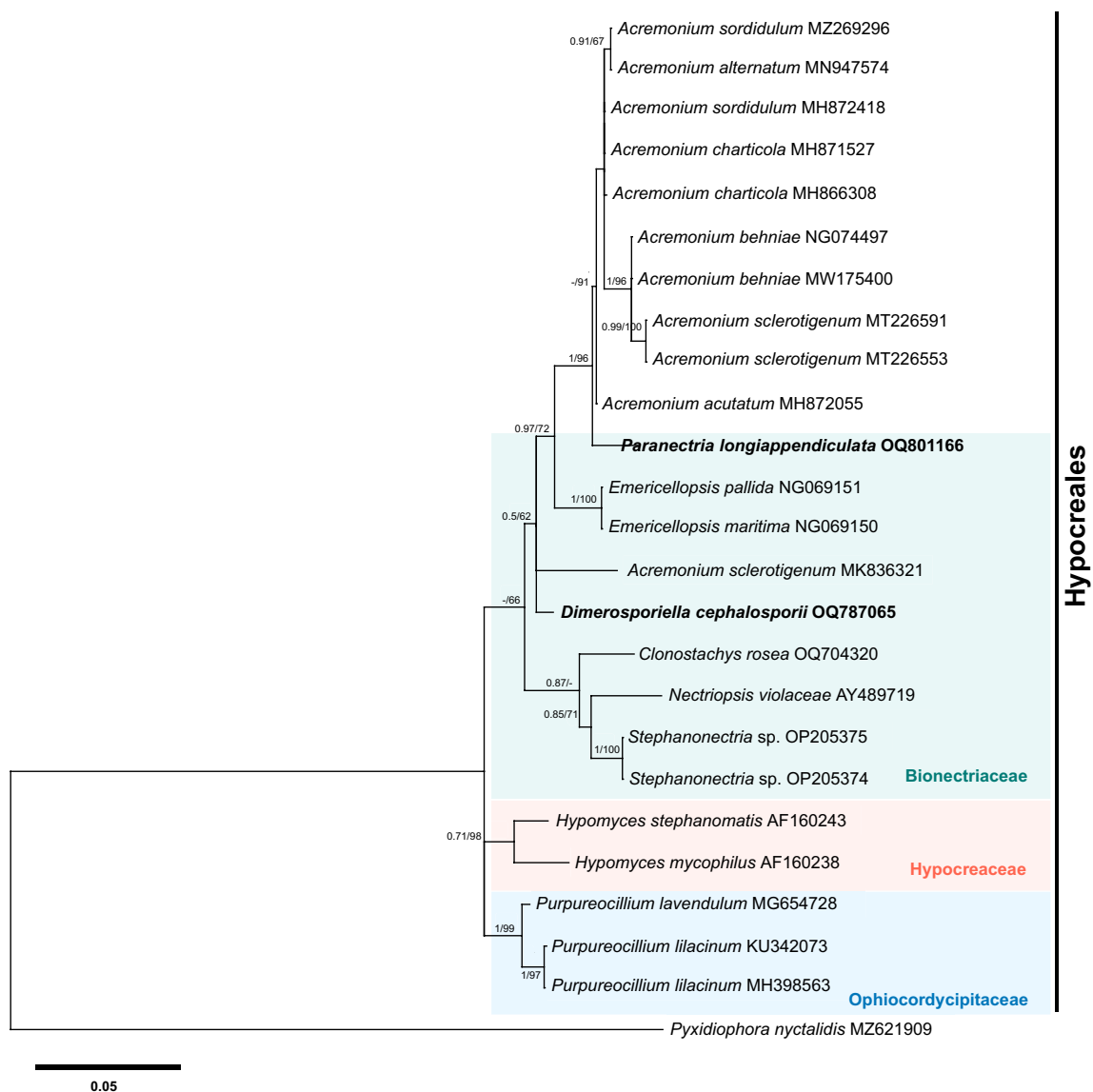
Fig. 4 Phylogenetic tree inferred from a maximum likelihood analysis of nuc LSU sequences of members of the *Bionectriaceae*, *Hypocreaceae*, and *Ophiocordycipitaceae* (*Hypocreales*), including a new sequence of *D. cephalosporii* and a new sequence of *Paranectria longiappendiculata*. The tree is rooted with *Pyxidiophora arvernensis* (*Pyxidiophoraceae*). Bootstrap values and posterior probabilities are indicated above the branches. Sequences downloaded from GenBank are given with accession numbers

Zalimey, Lama Forest, 6° 58′ 15″ N 2° 11′ 26″ E, 43 m a.s.l., 20 September 2022, A. Krauß, A. Tabé, N.S. Yorou, O. Koukol, AK20H (UNIPAR, M).

Known hosts and distribution – On *Meliola markhamiae* on living leaves of *Markhamia platycalyx* (*Bignoniaceae*) in Uganda (Hansford 1946); on *Meliola* sp. on living leaves of *Olyra latifolia* (*Poaceae*) in Panama (this study); on *Meliola pinnatae* on leaves of *Paullinia pinnata* (*Sapindaceae*) in Benin (this study). *M. pinnatae*, *O. latifolia*, and *P. pinnata* are new hosts of *D. cephalosporii*, and the hyperparasite is recorded here for mainland America (Panama) and Benin for the first time.

Illustrations – This species was illustrated by Gams (1971, anamorph only), Pirozynski (1977), and Samuels (1988).

Notes – Approximately 70 species of perithecioid fungi are reported as hyperparasites of *Meliolales* (Bermúdez-Cova et al. 2022). Among these species, *Dimerosporiella cephalosporii* (*Bionectriaceae*, *Hypocreales*) is one of the most common parasites in Uganda (Hansford 1946; Gams 1971; Gams et al. 2004; Bermúdez-Cova et al. 2022). The genus *Dimerosporiella* was proposed by Spegazzini (1908) and now comprises species that were previously placed in the *Nectria leucorrhodina* group or treated within *Nectriopsis* (Samuels 1976, 1988; Rossman 1983). Species of the genus are fungicolous (i.e., growing on other fungi) and grow on colonies of species of *Asterina*, *Meliolales*, or *Schiffnerula* (Rossman et al. 1999). Species of *Dimerosporiella* are differentiated

Fig. 5 *Dimerosporiella cephalosporii* (**MB86, MB139**). **a** A leaf of *Olyra latifolia* parasitized by *Meliola* sp. Note that some of the black colonies are whitish/greyish (arrows) due to the presence of the hyperparasite; **b** orange perithecia between the setae of *Meliola* sp.; **c** a leaf of *Paullinia pinnata* infected by *Meliola pinnatae* (MB139). Note that some of the black colonies are whitish/greyish due to the presence of *D. cephalosporii*; **d** perithecium on a hypha of *Meliola* sp.; **e** perithecial hairs; **f** ascus and ascospores. Scale bars: 1 cm (**a**, **c**); 1 mm (**b**); 13 μm (**d**); 5 μm; 4.5 μm (**f**)
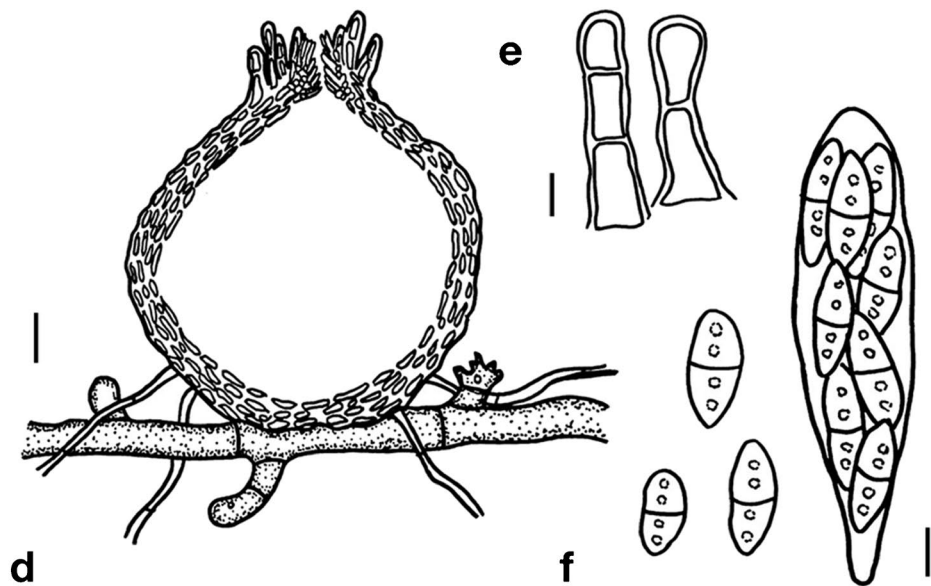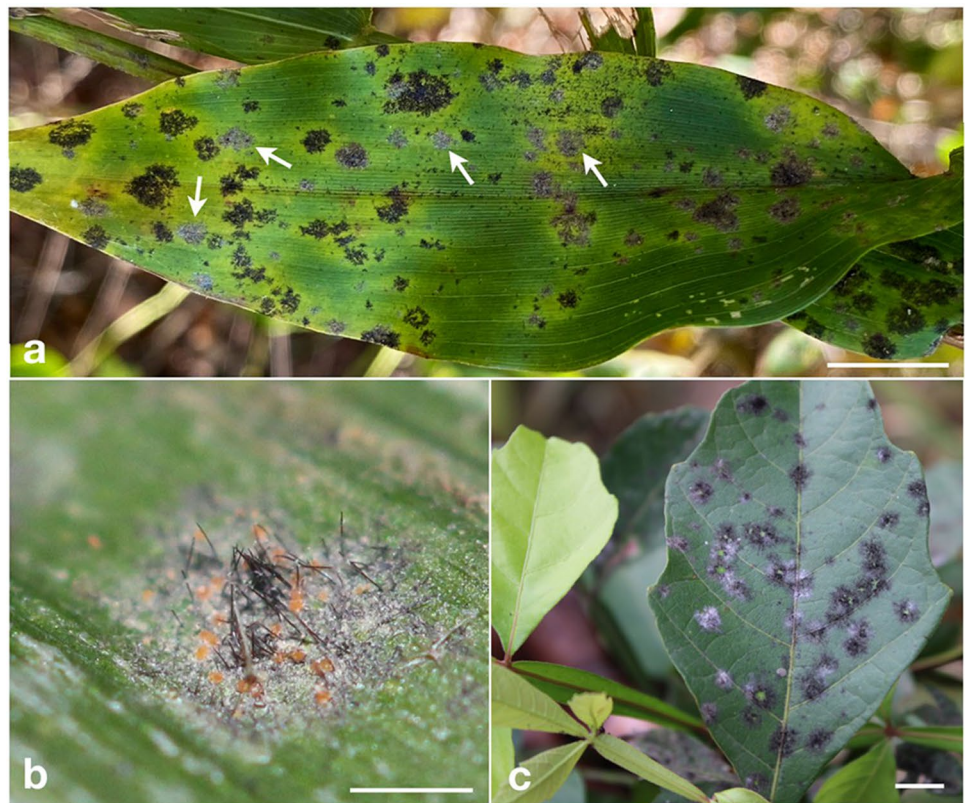
primarily by features of the surface of ascomatal walls and characteristics of the ascospores. For a detailed key to species of the genus, see Rossman et al. (1999).

Dimerosporiella cephalosporii is similar to *D. sensitiva*, from which it differs by a simple ascal apex and perithecial hairs (Samuels 1988). Both species are commonly associated with an *Acremonium*-like anamorph with thick-walled conidiophores and phialides (Pirozynski 1977). The conidial form is always found together with the perithecia, but there is no molecular evidence that supports this anamorph-teleomorph connection.

Sequence data – The LSU rDNA sequence obtained from fresh material of *D. cephalosporii* (specimen MB139) is 498 bp long and presented 32 ambiguous bases. In the tree

Fig. 6 The *Acremonium*-like anamorph of *Dimerosporiella cephalosporii* on *Meliola pinnatae* (MB139). **a**, **b** Conidiophores (arrows) with orange perithecia of *D. cephalosporii* on colonies of *Meliola pinnatae*; **c** conidiophore on a hypha of *Meliola* sp. and a young conidium; **d** conidia; **e** tip of a conidiophore with a young conidium. Scale bar: 3 μm (**c–e**)
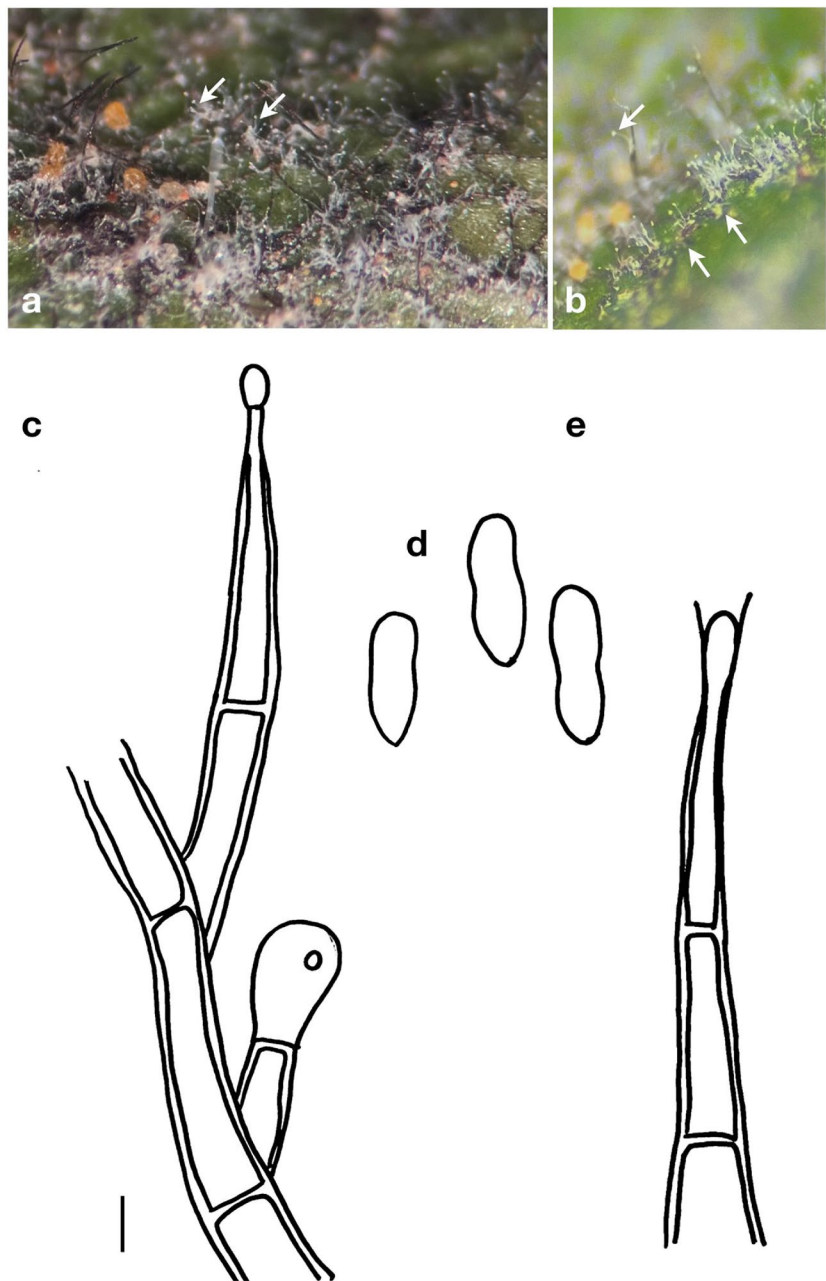
inferred from the analysis of LSU sequences of 24 specimens (Fig. 4), *D. cephalosporii* is located within a strongly supported clade that comprises sequences of *Acremonium* spp. and other species within the *Bionectriaceae*. It does not cluster with any sequence of *Dimerosporiella*, because no sequences are available for this genus up to now.

Malacaria meliolicola Syd., *Annls Mycol.* 28(1/2): 69, 1930 (Fig. 7).

≡ *Malacaria flagellata* (Hansf.) Hansf., *Mycol. Pap.* 15: 128, 1946.

≡ *Paranectria flagellata* Hansf., *Proc. Linn. Soc. London* 153(1): 28, 1941.

Colonies white, hyphae growing closely appressed to the dark hyphae of *Meliolales*, 1–2 μm wide, hyaline, thin-walled. Pseudothecia superficial, growing between the synnemata of *Atractilina parasitica*, ovate to elongate ovate with rounded apex, 150–200 × 100–140 μm, dark vinaceous when seen macroscopically, dark cinnamon or brick when seen by light microscopy, not changing color in KOH, smooth. Pseudothecial wall 12–17 μm thick, composed of angular cells with 6–15 μm diam. (surface view). Asci bitunicate, narrowly clavate to cylindric, apex rounded, (40–)52–56(–64) × (9.5–)10–12(–16) μm, 8-spored. Pseudoparaphyses unbranched, abundant, up to 120 μm long, 1–2 μm wide, septate, hyaline, rounded at the ends, with

a gelatinous external layer. Ascospores completely filling each ascus, mostly 3-septate, narrowly clavate, with an elongated base and rounded tips, (37–)44–54(–64)×3–4.5(–5) µm, pale smoke-grey, smooth.

Specimens examined – On *Meliola* sp. on leaves of *Coffea arabica*, Benin, Atlantique, Attogon, Niaouli Forest, 6° 44′ 42″ N 2° 7′ 50″ E, 69 m a.s.l., 28 February 2022, M.A. Bermúdez, A. Tabé, D. Dongnima, M. Piepenbring, N.S. Yorou, MB178; on *Meliola* sp. on living leaves of *Coffea arabica*, Benin, Atlantique, Attogon, Niaouli Forest, 6° 44′ 23″ N 2° 8′ 26″ E, 119 m a.s.l., 19 September 2022, A. Krauß, A. Tabé, N.S. Yorou, O. Koukol, AK4H (UNIPAR, M).

Known hosts and distribution – On *Irenina glabra* on leaves of *Coffea robusta* (*Rubiaceae*) in Uganda (Hansford 1941). On *Meliola* sp. on leaves of *Hamelia erecta* (*Rubiaceae*) in Venezuela (Rossman 1987). On *Meliola* sp. on leaves of *Coffea arabica* (*Rubiaceae*) in Benin (this study). *C. arabica* is a new host of *M. meliolicola*, and the hyperparasite is recorded here for Benin for the first time.

Illustrations – This species was illustrated by Rossman (1987).

Notes – *Malacaria meliolicola* (*Tubeufiaceae*, *Tubeufiales*) resembles other perithecioid species such as *Nematothecium vinosum* and *Hyalosphaera miconiae*, but it differs from these species by the presence of unbranched

Fig. 7 *Malacaria meliolicola* (**AK4H, MB178**). **a**, **b** Pseudothecia on black hyphae of *Meliola* sp. on living leaves of *Coffea arabica*; **c** pseudothecium on a hypha of *Meliola* sp.; **d** young and mature asci with pseudoparaphyses; **e** ascospores. Scale bars: approx. 500 µm (**a**); approx. 300 µm (**b**); 40 µm (**c**); 10 µm (**d**); 5 µm (**e**)
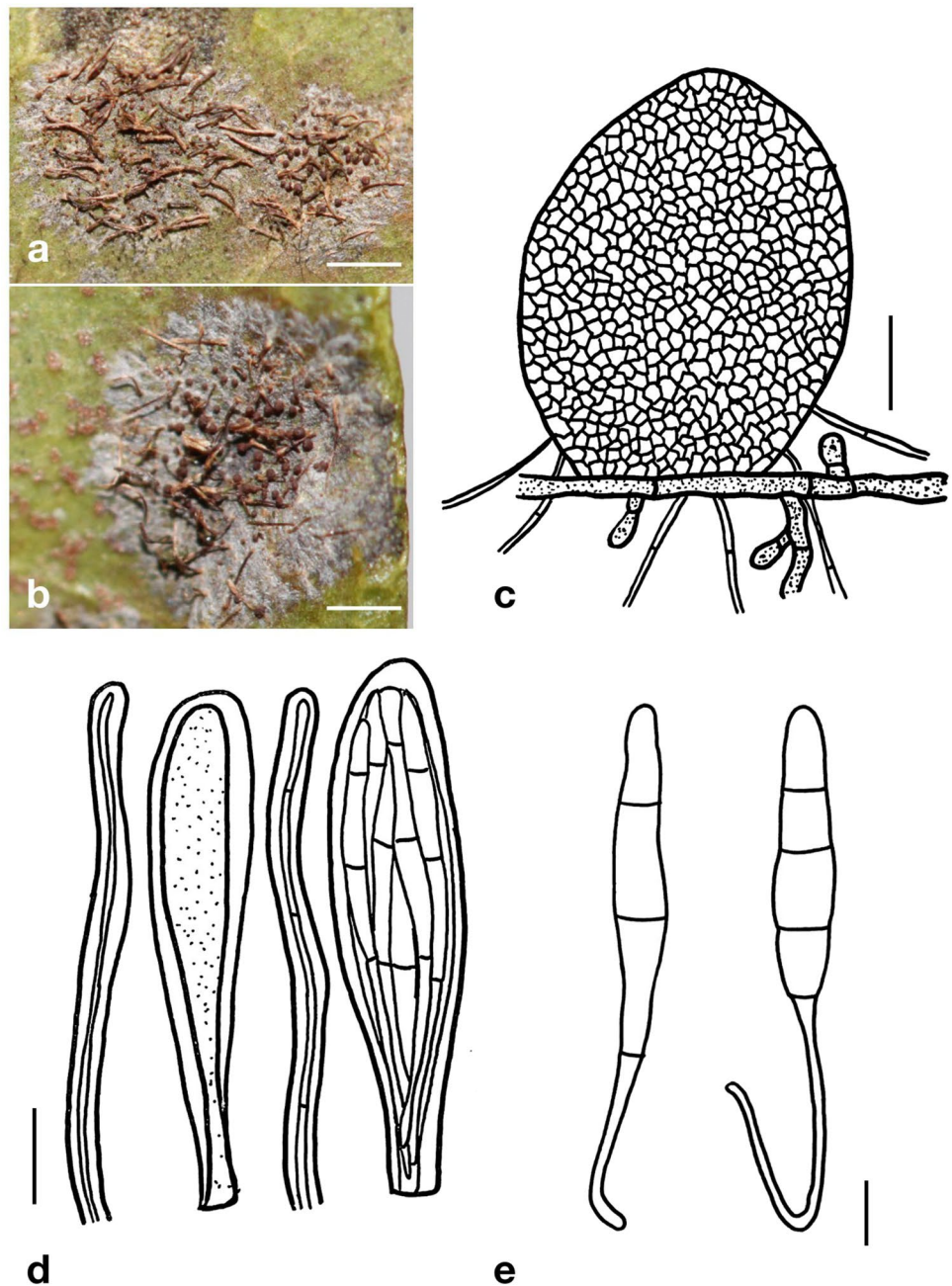
pseudoparaphyses (Rossman 1987). Hansford (1941, 1946) described *M. meliolicola* as the probable teleomorph of *Atractilina parasitica* (cited as *Arthrobotryum parasiticum*), a common hyperparasite of *Meliolales*. Apparently, the pseudothecia are only found when *A. parasitica* is present. However, there is no molecular evidence of this anamorph-teleomorph connection. According to Deighton and Pirozynski (1972), the connection is doubtful.

Paranectria longiappendiculata Berm.-Cova & M. Piepenbr., sp. nov. (Figs. 4 and 8).

MycoBank: MB#848317.

Fig. 8 *Paranectria longiappendiculata* (**MB175**). **a** Perithecia on black hyphae of *Meliola* sp.; **b** perithecium, as seen by light microscopy; **c** A perithecium on hyphae of *Meliola* sp. Left side: cross section view, right side: surface view; **d** perithecial hairs; **e** ascospores. The thickness of the walls is shown for two spores. Scale bars: 3 mm (**a**); 36 μm (**b**); 20 μm (**c**); 5 μm (**d**, **e**)
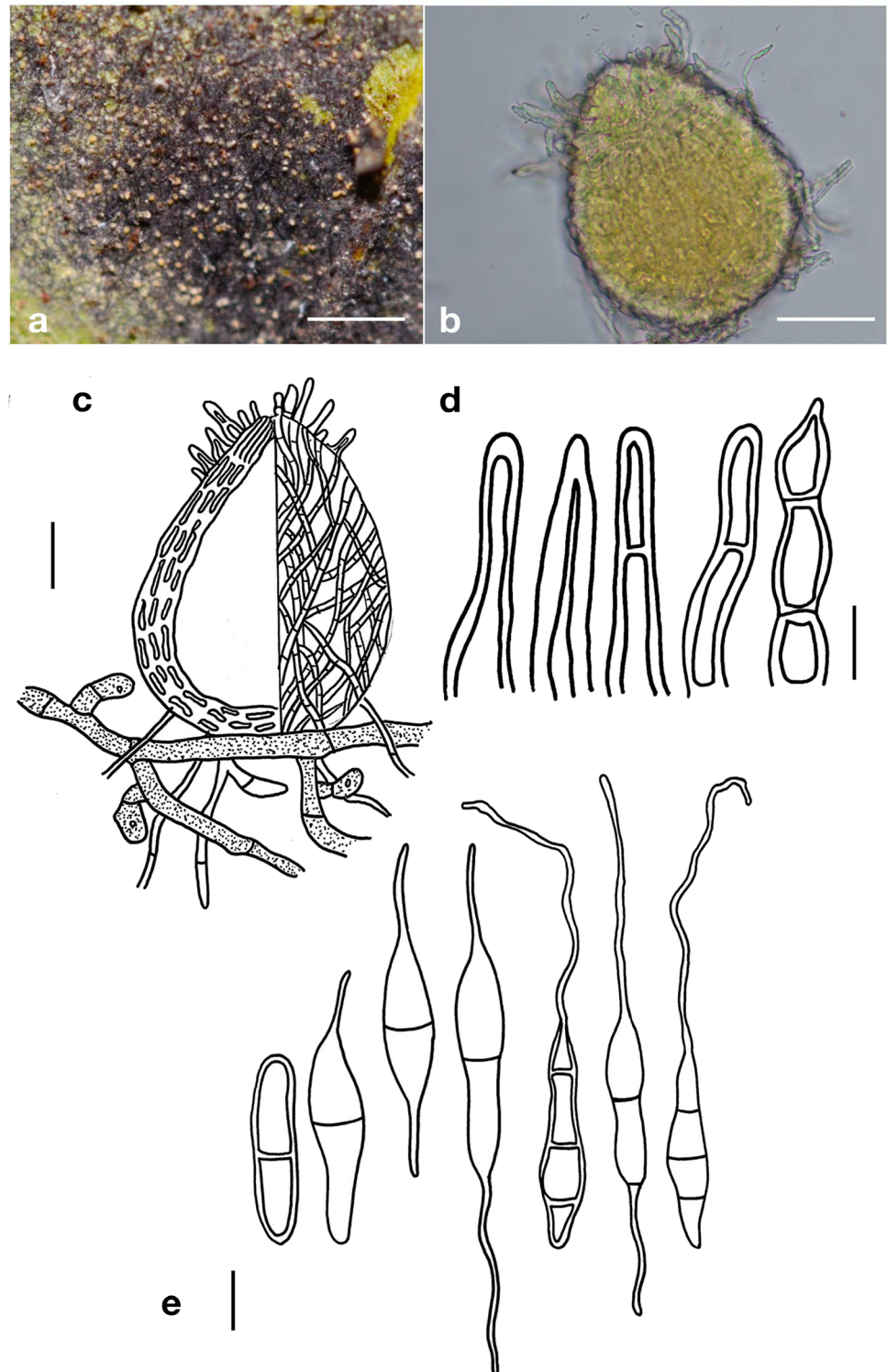
Holotype – On *Meliola* sp. on living leaves of *Angylocalyx oligophyllus*, Benin, Atlantique, Attogon, Niaouli Forest, 6° 44′ 42″ N 2° 7′ 50″ E, 69 m a.s.l., 28 February 2022, M.A. Bermúdez, A. Tabé, D. Dongnima, I. Agonglo, O.P. Agbani, M. Piepenbring, N.S. Yorou, MB175 (M, GenBank accession number: OQ801166).

Paratype – Same locality, collection date, fungal and plant hosts, MB169 (UNIPAR).

Etymology – Named for the long appendages of the ascospores.

Colonies of white thin hyphae covering the colonies of *Meliolales*. Hyphae thin-walled, septate, 1–2 μm wide, hyaline. Perithecia solitary or in small groups, scattered, superficial, ovate to elongate ovate with rounded apex, (70–)90–104(–113) μm diam., pale orange to orange, not changing color in KOH, with ascomatal hairs mostly around the apex. Hairs straight to crooked, non-septate or septate, unbranched, apex obtuse or pointed, 14–20×2–4 μm, hyaline. Ascomatal wall 10–13 μm wide, composed of elongated cells parallel to the inner surface of the perithecium as seen in longitudinal section, and of loosely interwoven septate hyphae (surface view). Asci not found. Ascospores fusiform to ellipsoid, (12–)16–21(–32)×2–4 μm (measurements without appendages), 1–3-septate, hyaline, smooth, with straight or curved appendages at one or both tips (rarely without appendages), up to 40 μm long. Ascospores tend to stick together when liberated from the perithecia. Ascospores tend to separate from each other when KOH is added.

Anamorph – Not known.

Known distribution – On colonies of *Meliola* sp. on living leaves of *Angylocalyx oligophyllus* (*Fabaceae*) in Benin.

Notes – The genus *Paranectria* was proposed by Saccardo (1878), with *P. affinis* as type species, a wood-inhabiting species of the *Hypocreales* (*Sordariomycetes*). The genus initially comprised species with hyaline, 3-septate ascospores that carry appendages at both tips (Rossman 1987). Based on this description, Stevens (1918), Hansford (1941, 1946) and other authors proposed new species in this genus, all with fungicolous lifestyle. However, none of these authors seemed to notice that many of these fungi have bitunicate asci, a feature that is present in the *Dothideomycetes* and not in the *Sordariomycetes*. Therefore, Pirozynski (1977) transferred many of these species to the genus *Paranectriella* (P. Henn.) Piroz., a genus that comprises eight species of tropical hyperparasites of plant parasitic fungi that resemble *Paranectria*, but differ fundamentally in possessing bitunicate asci. In addition to this, cells of perithecial walls of species of *Bionectriaceae* and *Nectriaceae* (*Hypocreales*) typically are thin-walled and elongated parallel to the surface of the perithecia as seen in longitudinal sections (Rossman et al. 1999), while corresponding cells of species of *Paranectriella* are isodiametric (see the examples of *Paranectriella hemileiae* and *Paranectriella minuta* below).

Paranectria longiappendiculata (specimens MB169, MB175) resembles species of the genera *Paranectria* and *Paranectriella* by the fungicolous lifestyle and partly 3-septate ascospores with appendages at the tips. In comparison to *Paranectria affinis* (spores 24–34 μm long; Saccardo 1878), the ascospores of *P. longiappendiculata* are shorter (up to 21 μm long). *Paranectriella hemileiae* and *Paranectriella minuta* produce hairs on the surface of the ascomata like *P. elongata*, but *P. elongata* differs by ascospores with long terminal appendages that can reach a length of up to 40 μm. Appendages of all the other known species of *Paranectria* and *Paranectriella* only reach up to 20 μm (Saccardo 1878; Rossman 1987). Asci were not found in the examined specimens, so it is not possible to assign them to *Sordariomycetes* or *Dothideomycetes* based on details of the walls of asci. The cells of the asci of *P. longiappendiculata*, however, resemble those of species of hypocrealean fungi within the Bionectriaceae and Nectriaceae (Rossman et al. 1999).

Sequence data – The LSU rDNA sequence obtained from fresh material of *P. longiappendiculata* (specimen MB175) is 811 bp long. Based on a MegaBLAST search in the NCBI GenBank nucleotide database using the LSU sequence data of *P. longiappendiculata*, the closest match was *Acremonium acutatum* (GenBank MH872055; identities 726/799, i.e., 90.86%), as well as other species of hypocrealean fungi. The morphological features discussed above, together with the results of the MegaBLAST search, confirm the placement of *P. longiappendiculata* in the *Hypocreales* and in the genus *Paranectria*. In the tree inferred from the analysis of LSU sequences of 24 specimens (Fig. 4), *P. longiappendiculata* is located within a strongly supported clade that comprises sequences of *Acremonium* spp. and other species within the *Bionectriaceae*.

Paranectriella hemileiae (Hansf.) Piroz., *Kew Bull.* 31: 598, 1977 (Fig. 9).

 ≡ *Paranectria hemileiae* Hansf., *Proc. Linn. Soc. Lond.* 153: 28, 1941.

Colonies of white hyphae spreading over the colonies of *Meliola* sp. Pseudothecia solitary, scattered, superficial, globose to subglobose, 130–180 μm diam., pale luteous to white, not changing color in KOH, with sparse to abundant ascomatal hairs, scattered all over the ascomatal surface. Hairs straight to slightly sigmoid, septate or non-septate, unbranched, thick-walled, 14–30×4–6 μm, hyaline. Pseudothecial wall composed of isodiametric cells, 5–9 μm, thin-walled (surface view). Asci bitunicate, clavate to broadly cylindric, apex rounded, 50–68×9–14 μm, 8-spored. Pseudoparaphyses not seen. Ascospores fusiform, mostly 3-septate, slightly constricted at the septa, with straight appendages mostly at both tips, (14–)16–18(–20)×5–7 μm, hyaline, smooth.

Fig. 9 *Paranectriella hemileiae*
(**MB108**). **a** Pseudothecium on
a hypha of *Meliola* sp. (content
not drawn); **b** perithecial hairs;
c ascus with ascospores; **d**
ascospores. Scale bars: 25 μm
(**a**); 5 μm (**b**); 10 μm (**c**); 3 μm
(**d**)
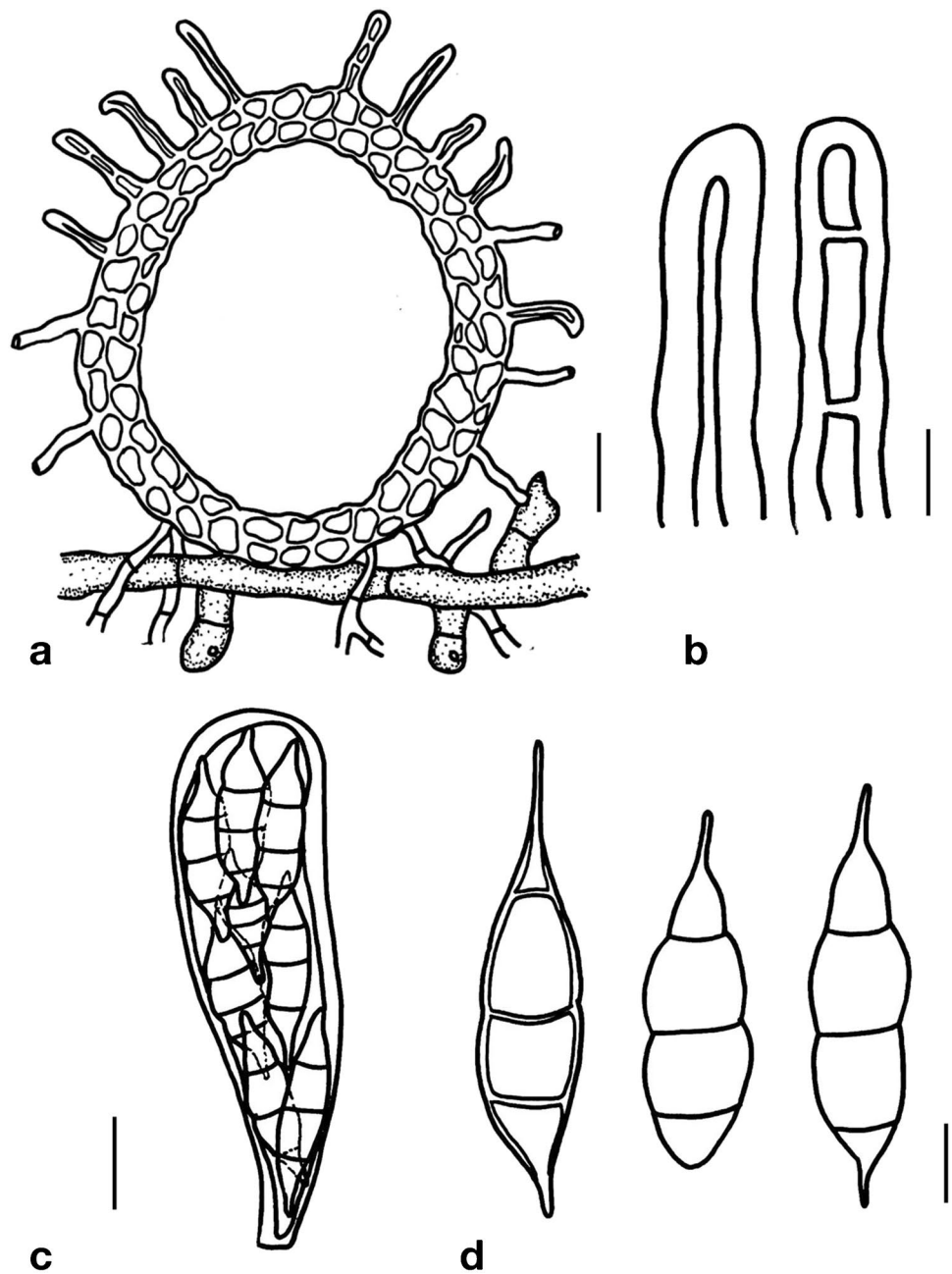
Anamorph – Not observed (*Titaea hemileiae* Hansf. according to Rossman 1987).

Specimen examined – On *Meliola* sp. on living leaves of *Xylopia frutescens*, Panama, Chiriquí Province, Cochea, Cochea river trail, 8° 32′ 37″ N 82° 23′ 03″ W, 181 m a.s.l., 26 February 2020, M.A. Bermúdez, A. Sanjur, A. Villarreal, MB108 (UCH13409).

Known hosts and distribution – On sori of *Hemileia vastatrix* (*Pucciniales*) on leaves of *Coffea robusta* (*Rubiaceae*) in Uganda (Rossman 1987); on *Meliola* sp. on leaves of *Xylopia frutescens* (*Annonaceae*) in Panama (this study). *Meliola* sp. and *X. frutescens* are new hosts of *P. hemileiae*,

and the hyperparasite is recorded here for mainland America (Panama) for the first time.

Illustrations – This species was illustrated by Pirozynski (1977) and Rossman (1987), as well as by Carmichael et al. (1980, anamorph only) and Hansford (1946, anamorph only).

Notes – Up to now, the sexual form *Paranectriella hemileiae* is only known from the type specimen, growing on sori of *Hemileia vastatrix*. Despite its occurrence on a rust, the species is retained in the genus *Paranectriella* due to the presence of 3-septate ascospores with terminal appendages (Rossman 1987). There is a possible associated

anamorph to this species, namely, *Titaea hemileiae*. It produces staurospores, like some other species (e.g., *P. miconiae*; Pirozynski 1977, Rossman 1987). However, no conidia were found in the examined specimen (MB108).

Paranectriella minuta (Hansf.) Piroz., *Kew Bull.* 31(3): 600, 1977 (Fig. 10).

≡ *Paranectria minuta* Hansf., *Proc. Linn. Soc. London* 153(1): 30, 1941.

Colonies of white hyphae covering colonies of *Meliolales*. Hyphae thin-walled, septate, 2–3 μm wide, hyaline. Pseudothecia solitary or in small groups, scattered, superficial, globose, (80–)90–115(–150) μm diam., pale luteous, pale orange to white, translucent, not changing color in KOH, with ascomatal hairs more or less close to the apex. Hairs straight to crooked, unbranched, apex obtuse, non-septate, 24–40 × 3–6 μm, hyaline. Pseudothecial wall 6–10 μm thick, composed of isodiametric cells 7–15 μm wide, thin-walled (surface view). Asci bitunicate, broadly cylindric to obovate, apex rounded, (37–)40–50(–61) × 12–18 μm, 8-spored. Pseudoparaphyses not seen. Ascospores fusiform to ellipsoid, 3-septate, slightly constricted at the septa, with a straight or curved appendage of 3–11 μm length at each tip, (14–)16–18 × 5–6 μm, hyaline, smooth.

Specimens examined – On *Meliola* sp. on living leaves of *Coffea arabica*, Benin, Atlantique, Attogon, Niaouli Forest, 6° 44′ 23″ N 2° 8′ 26″ E, 119 m a.s.l., 19 September 2022, A. Krauß, A. Tabé, N.S. Yorou, O. Koukol, AK4H (M); on *Meliola* sp. on living leaves of *Opilia celtidifolia*, Benin, Donga, Bassila, 8° 59′ 58″ N 1° 38′ 45″ E, 360 m a.s.l., 27 September 2022, A. Krauß, A. Tabé, N.S. Yorou, O. Koukol, AK38H (UNIPAR, GenBank accession number: OQ801179).

Known hosts and distribution – On *Meliola paulliniae* on leaves of *Paullinia pinnata* (*Sapindaceae*) in Uganda (Hansford 1941); on *Meliola* sp. on leaves of *Coffea arabica* (*Rubiaceae*) in Benin (this study); on *Meliola* sp. on leaves of *Opilia celtidifolia* (*Opiliaceae*) in Benin (this study). *C. arabica* and *O. celtidifolia* are new hosts of *P. minuta*, and the hyperparasite is recorded here for Benin for the first time.

Anamorph – Not known.

Illustrations – This species was illustrated by Hansford (1941), Pirozynski (1977) and Rossman (1987).

Notes – *Paranectriella minuta* is similar to *P. hemileiae*, but the ascomatal hairs of *P. minuta* are located mostly close to the apex of the pseudothecium. The presence of appendages on the ascospores and small, translucent ascomata can also occur in some species of the genus *Hyalocrea*, but *Hyalocrea* spp. are characterized by the absence of pseudoparaphyses (Rossman 1987). Pseudoparaphyses, however, were not found in the specimen examined (AK38H). Nevertheless,

we identify the specimens from Benin as *P. minuta*, because the ascospores of these specimens are smaller than those of hyperparasitic species of *Hyalocrea* (e.g., *H. meliolicola*, 26–35 × 7–9 μm; Rossman 1987).

Sequence data – The LSU rDNA sequence obtained from fresh material of *P. minuta* (specimen AK38H) is 494 bp long. Based on a MegaBLAST search in the NCBI GenBank nucleotide database using the LSU sequence data of *P. minuta*, the closest match was *Quixadomyces hongheensis* (GenBank MW264194; identities 460/491, i.e., 93.69%), as well as other species of *Pleosporales*. Hyde et al. (2013) designated the family *Paranectriellaceae* to accommodate hyperparasitic species of *Dothideomycetes* with bright colored ascomata, ascospores with transverse septa and prominent appendages. However, there is no molecular DNA sequence data that supports this designation. Ours represent the first DNA sequence of a fungus of the genus *Paranectriella*, and more sequences are necessary to evaluate this hypothesis.

Key to species of perithecioid hyperparasites on *Meliolales* known for Benin and Panama

1 Ascomata dark vinaceous to dark brick; ascospores smoke-gray.. ***Malacaria meliolicola***
1* Ascomata white, pale luteous to orange; ascospores hyaline... 2
2 Ascospores 1-septate, biguttulate; asci unitunicate.................................... ***Dimerosporiella cephalosporii***
2* Ascospores (up to) 3-septate, with appendages at their tips; asci bitunicate... 3
3 Appendages 20–40 μm long... .... ***Paranectria longiappendiculata***
3* Appendages up to 20 μm long.. 4
4 Pseudothecia with sparse to abundant thick-walled ascomatal hairs, scattered all over the ascomatal surface.............. ....***Paranectriella hemileiae***
4* Pseudothecia with ascomatal hairs mostly close to the apex ***Paranectriella minuta.***

Discussion

Hyperparasitic fungi on *Meliolales* have been collected in the past mainly in Brazil, Dominican Republic, and Puerto Rico in America, as well as in Ghana, Sierra Leone, and Uganda in Africa (Bermúdez-Cova et al. 2022). In the context of the present study, we analyzed 16 specimens of *Meliolales* associated with hyperparasites, corresponding to eight species of hyperparasitic fungi. Seven species represent new records: five for Benin and four for Panama. One species is new to science. *Calloriopsis herpotricha* is recorded for the first time for Africa and *Dimerosporiella cephalosporii*

Fig. 10 *Paranectriella minuta (AK4H, AK38H)*. **a** Pseudothecia on black hyphae of *Meliola* sp. on a living leaf of *Coffea arabica*; **b** pseudothecium on hyphae of *Meliola* sp.; **c** ascus with ascospores; **d** ascospores. Scale bars: 500 μm (**a**); 15 μm (**b**); 10 μm (**c**); 3 μm (**d**)
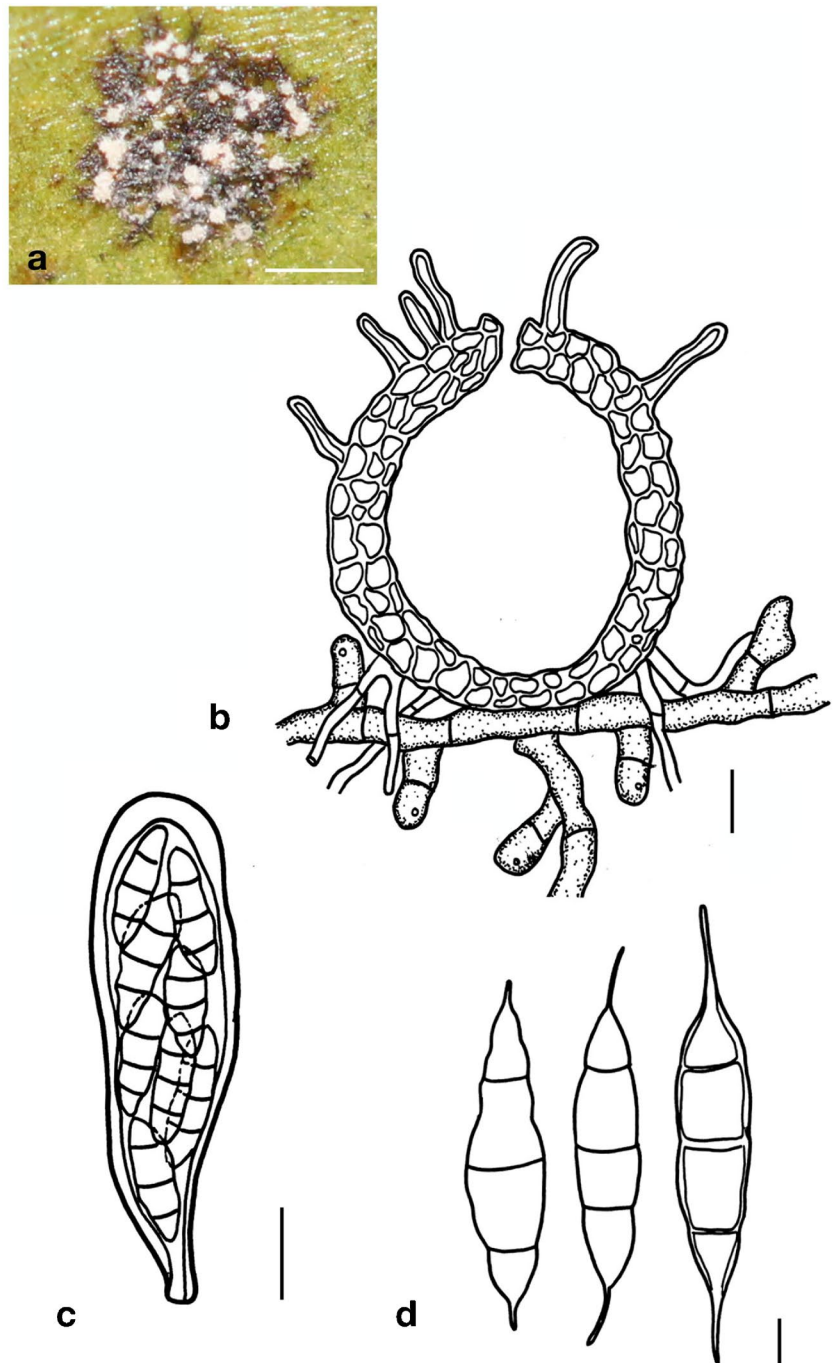
and *Paranectriella hemileiae* for mainland America. These findings are based on only three months of fieldwork and show a blatant lack of investigation on hyperparasitic fungi in the tropics.

Patterns of distribution of hyperparasitic fungi have been studied mainly for hyperparasites of rusts and powdery mildews (Zewdie et al. 2021), but never for those infecting black mildews. The distribution of hyperparasitic fungi is restricted to that of their host (Sun et al. 2019). As *Meliolales* are restricted to tropical and subtropical areas (Piepenbring 2015), hyperparasites are expected to be found in these regions as well. We also expect wide distribution areas of hyperparasitic fungi on *Meliolales* because of their broad spectra of host species (Bermúdez-Cova et al. 2022). In fact, the data presented in this study suggest that at least part of the species of hyperparasitic fungi of *Meliolales* have a pantropical distribution, as they have been recorded both in paleotropical and neotropical regions. This is consistent with the assumptions made by Samuels et al. (2002) regarding the pantropical distribution

of tropical perithecioid fungi. Extensive additional fieldwork is needed in order to unravel distribution patterns of hyperparasitic fungi on meliolalean hosts.

It is difficult to obtain molecular sequence data from hyperparasites especially because of their incapability of growing in artificial media and the fact that they develop intermingled with the primary parasite and many other organisms (Bermúdez-Cova et al. 2022). As a consequence, isolating and sequencing hyperparasitic fungi is a challenging task. There is also a lack of sequences of hyperparasitic fungi in public databases. Therefore, the sequences obtained in the context of the present work can be related to existing species concepts only based on morphology, and issues such as anamorph-teleomorph connections cannot be confirmed. Nevertheless, in this study for the first time ever, DNA sequences of hyperparasitic fungi on *Meliolales* are published. This example emphasizes that field work paired with molecular analysis still plays a crucial role for modern mycology, especially for challenging fungal groups, such as hyperparasites.

Declarations

Competing interests The authors declare no competing interests.

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Anlage 4

Erklärung zu den Autorenanteilen an dem Manuskript (Titel):

Systematic revision of species of *Atractilina* and *Spiropes* hyperparasitic on Meliolales in the tropics

Status: submitted. Under review

Beteiligte Autoren:

- MABC: Miguel A. Bermúdez-Cova

- TAH: Tina A. Hofmann

- NSY: Nourou S. Yorou

- MP: Meike Piepenbring

Was hat der Promovierende bzw. was haben die Koautoren beigetragen?

(16)   zu Entwicklung und Planung

Promovierender MABC: 80 %

Koautor TAH: 5 %

Koautor NSY: 5 %

Koautor MP: 10 %

(17)   zur Durchführung der einzelnen Untersuchungen und Experimente

Promovierender MABC: 100 %

MABC carried out the DNA extraction, amplification (PCR) and sequencing.

(18)   zur Erstellung der Datensammlung und Abbildungen

Promovierender MABC: 85 %

Koautor TAH: 5 %

Koautor NSY: 5 %

Koautor MP: 5 %

MABC collected most of the samples in Panama and Benin in 2020 and 2022, respectively and prepared the figures plates. Plates and illustrations were made by MABC. TAH, NSY collaborated with host plant identification. TAH and NSY collaborated with logistics and sampling in Panama and Benin. MP provided some pictures of the fungi in the field.

(19)   zur Analyse und Interpretation der Daten

Promovierender MABC: 80 %

Koautor TAH: 5 %

Koautor NSY: 5 %

Koautor MP: 10 %

MABC performed DNA sequence analyses, multiple sequence alignments and phylogenetic reconstructions. Interpretation of the phylogenies from a systematic point of view was done by MABC in collaboration with TAH, NSY and MP. Nomenclatural decisions were taken by MABC and MP.

(20)   zum Verfassen des Manuskripts

Promovierender MABC: 80 %

Koautor TAH: 5 %

Koautor NSY: 5 %

Koautor MP: 10 %

Zustimmende Bestätigungen der oben genannten Angaben:

Datum/Ort   Unterschrift Promovend

Datum/Ort   Unterschrift Betreuer

Datum/Ort   Ggfs. Unterschrift *corresponding author*

Systematic revision of species of *Atractilina* and *Spiropes* hyperparasitic on Meliolales in the tropics

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Abstract

Atractilina Dearn. & Barthol. and *Spiropes* Cif. are genera of asexual fungi that comprise species mainly hyperparasitic on black mildews (Meliolales, Ascomycota). Although a common group of anamorphic fungi, they have been described up to now only by morphology, and their systematic position is unknown. The present study provides a morphological treatise of all known species of *Atractilina* and *Spiropes* hyperparasitic on Meliolales, including insights in their systematic position based on DNA sequences generated here for the first time. The study was conducted based on 33 herbarium specimens and 23 specimens recently collected in Benin and Panama. The obtained DNA sequence data (28S rDNA and ITS rDNA) of *A. parasitica* and of two species of *Spiropes* show systematic placements in the Dothideomycetes and Leotiomycetes, respectively. The sequence data of the two *Spiropes* spp. do not group together. Moreover, the anamorph-teleomorph connection between *Atractilina parasitica* and *Malacaria meliolicola*, a pseudothecioid fungus, is confirmed. Three species in the genus *Spiropes* are proposed as new to science, namely *S. angylocalycis, S. carpolobiae* and *S. croissantiformis*. Four species are reported for Benin for the first time, three species for Panama and one species for mainland America. *Atractilina* and *Spiropes* are currently two genera with highly heterogeneous species, and they might have to be split in the future, once the taxonomic concepts are validated by morphology and molecular sequence data.

Introduction

Meliolales (Sordariomycetes, Ascomycota) form a large order of biotrophic, obligate plant parasitic fungi in the tropics and subtropics (Piepenbring et al. 2011; Hongsanan et al. 2015; Zeng et al. 2017). The order comprises two families, Armatellaceae and Meliolaceae, with *Armatella* Theiss. & Syd. and *Meliola* Fr. being the most species-rich genera of each family, respectively (Hosagoudar 2003; Jayawardena et al. 2020). They are commonly known as "black mildews", because they produce black colonies that are composed of dark, thick-walled, branched, superficial hyphae (Rodríguez Justavino et al. 2015).

Approximately 200 species of hyperparasitic fungi, i.e., fungi parasitic on other parasites, have been reported to grow on colonies of Meliolales (Bermúdez-Cova et al. 2022, 2023a). These hyperparasites mainly belong to the Dothideomycetes and the Sordariomycetes, although the systematic positions of a large number of these fungi still remain unknown (Bermúdez-Cova et al. 2022, Bermúdez-Cova et al. 2023a). Hyperparasitic fungi frequently overgrow entire colonies of black mildews so the meliolalean host may be detected only by careful search with a light microscope (Stevens 1918; Ciferri 1955; Bermúdez-Cova et al. 2023a).

Amongst the hyperparasitic fungi, species of the anamorphic genera *Atractilina* Dearn. & Barthol. and *Spiropes* Cif. are common hyperparasites of black mildews in the tropics. In the past, they were regarded as conidial stages of Meliolales (Ciferri 1955; Bermúdez-Cova et al. 2023b), and nowadays as incertae sedis in the Ascomycota (Bermúdez-Cova et al. 2022). The genus *Atractilina* includes six species of mostly hyperparasitic hyphomycetes with true synnemata, denticulate conidiogenous loci and pale pluriseptate conidia (Deighton and Pirozynski 1972; Mel'nik and Braun 2013). On the other hand, the genus *Spiropes* comprises 34 species of dematiaceous, mostly hyperparasitic hyphomycetes with mononematous, fasciculate or synnematous conidiophores (Ellis 1968, 1971, 1976; Seifert and Hughes 2000; Bánki et al. 2023). Species of *Spiropes* are characterized by the presence of conidiogenous cells with conspicuous, flat and numerous scars, as well as pigmented conidia with 1–9 septa or pseudosepta (Ellis 1968).

Arthrobotryum Ces., *Cercospora* Fresen. ex Fuckel, *Helminthosporium* Link, *Pleurophragmium* Costantin and *Podosporium* Schwein. are only a few of the many genera to which species of *Atractilina* and *Spiropes* have been assigned in the past, although they were not congeneric with the type specimens of those genera (Ellis 1968; Deighton and Pirozynski

1972; Alcorn 1988). This resulted in taxonomic uncertainty with species being transferred from one genus to another. This problem was initially addressed by Ellis (1968) and Deighton and Pirozinsky (1972), as they did an extensive morphological revision of taxa now assigned to *Atractilina* or *Spiropes.* For example, all the synnematous fungi hyperparasitic on Meliolales formerly assigned to the genus *Arthrobotryum*, were transferred to the genus *Spiropes* by Ellis (1968), with the exception of *A. parasiticum* (Winter) Hansf., which was transferred to the genus *Atractilina* by Deighton and Pirozynski (1972).

There is currently one valid species of *Atractilina*, namely *A. parasitica* (G. Winter) Deighton & Piroz., and 19 species of the genus *Spiropes* known to be hyperparasitic on colonies of Meliolales (Ellis 1968; Deighton and Pirozynski 1972; Mel'nik and Braun 2013; Bermúdez-Cova et al. 2022). However, species delimitation within these two genera has up to now been done by morphology only, as species were described in the past before the molecular era, and because of the challenges of isolating DNA from mixed infections (Bermúdez-Cova et al. 2022, 2023a, 2023b). As a result, the systematic position of both genera within the Ascomycota remained unknown. The present study revises the morphology of the species of *Atractilina* and *Spiropes*, and provides the first insights in their systematic position according to molecular sequence data, with emphasis on the species hyperparasitic on Meliolales.

Materials and methods

Sample collection and morphological characterization

Samples of leaves infected with black mildews were opportunistically collected in Western Panama from January-March 2020 and in Benin in February as well as September-October 2022. For the present study, colonies of Meliolales hyperparasitized by *Atractilina parasitica* and species of *Spiropes* were considered. Infected leaves were dried in a plant press and deposited in the herbarium at the Universidad Autónoma de Chiriquí (UCH, specimens from Panama) or in the mycological herbarium of the University of Parakou (UNIPAR) in Benin. Duplicates of large-sized samples were deposited in the Botanische Staatssammlung München (M).

Dried specimens were observed by stereomicroscopy and by light microscopy (LM). Measurements of at least 20 conidia and other structures have been made for each specimen at magnifications of ×600 and ×1000. Measurements are presented as mean value ± standard deviation with extreme values in parentheses. Line drawings were made freehand on scaled

paper. Scars on conidiophores are drawn in surface view although further cells of the conidiophore are drawn in optical section. Images and drawings were edited with Photoshop (Adobe, San Jose, California). Specimens were also analyzed morphologically by scanning electron microscopy (SEM). Materials used for SEM were prepared according to Hofmann et al. (2010).

Host plant identification

Host plants were identified by morphological characteristics and, in some cases, by molecular sequence data. Morphological identifications were made by comparison with herbarium specimens, literature (e.g., Akoègninou et al. 2006; Condit et al. 2011) and with the help of local botanists. Molecular sequence data for species identifications were obtained by polymerase chain reaction (PCR) for the amplification of the partial region of chloroplast rbcL with the primer pairs rbcLa-F (Levin et al. 2003) and rbcLa-R (Kress et al. 2009). DNA was extracted from approx. 0.05 g of leaf tissue dried with silica gel using the innuPREP Plant DNA Kit (Analytik Jena, Germany) and following the manufacturer's instructions. Protocols for PCR were carried out as described by Fazekas et al. (2012).

DNA extraction, PCR amplification and sequencing of fungal DNA

DNA was isolated from the synnemata and hyphae of dry specimens using the E.Z.N.A Forensic DNA Extraction Kit, following the manufacturer's instructions. To extract total genomic DNA, a small amount of clean synnemata or single conidiophores were transferred into a sterile Eppendorf tube with approx. 200 μL of distilled water using sterilized tweezers, and trying to avoid picking cells of any other organism associated with the leaves and the colonies of black mildews. The samples were frozen for 24 h at -20 °C, and later homogenized for 10–12 min. using a Retsch Mixer Mill MM301 with TL buffer and 2.5 mm Zirconia beads. Isolated DNA was re-suspended in elution buffer and stored at -20 °C.

Two partial nuclear gene regions (ribosomal loci) were amplified and sequenced: For the large subunit nuclear ribosomal DNA (nrLSU, 28S rDNA) the primers LR0R (Wagner and Ryvarden 2002) and LR5 (Vilgalys and Hester 1990) were used. For the internal transcribed spacer region of ribosomal DNA (ITS), the primers ITS5 and ITS4 (White et al. 1990) were used. The PCR mixtures consisted of 1 μL genomic DNA, 15× $MgCl_2$ reaction buffer (Bioline, Luckenwalde, Germany), 25 mM $MgCl_2$, 25 μM of each dNTP, 10 μM of each primer and 5 U Taq DNA polymerase (VWR) in a total volume of 30 μL. Cycling parameters of the PCR were as follows:

initial denaturation at 94 °C for 3 min, followed by 35 cycles of amplification [denaturation at 94 °C for 30 s, primer annealing at 52 °C for 30 s and primer extension at 72 °C for 45 s] and a final extension at 72 °C for 5 min, followed by storage at 8 °C. PCR-products were checked on 1.5% agarose electrophoresis gels containing HDGreenPlus DNA stain. Amplified PCR products were purified with the Cycle Pure Kit (VWR-Omega, USA). Sequencing was performed at Seqlab GmbH, Germany.

Phylogenetic analyses

Consensus sequences of trace files were generated with Geneious 10.2.2 (https://www.geneious.com, Kearse et al. 2012) and searched against GenBank (https://www.ncbi.nlm.nih.gov/, Benson et al. 2014) with MegaBLAST. Ambiguous and miscalled bases were corrected, when possible, after examination of the corresponding chromatogram files. Sequences with a high similarity were aligned with MAFFT v. 7 using the L-INS-i algorithm (Nakamura et al. 2018). The alignments were manually checked by using MEGA v. 7 (Kumar et al. 2016). Gblocks v. 0.91b (Talavera and Castresana 2007) was used to remove poorly aligned positions and divergent regions from the DNA alignment. Phylogenetic analyses of this study were conducted by applying Maximum Likelihood (ML) in RAxML-HPC2 v.8.2.12 (Stamatakis 2014) on XSEDE (Miller et al. 2010) and Bayesian phylogenetic inference with the program MrBayes 3.2.6. (Ronquist et al. 2012) on XSEDE (Miller et al. 2010), available on the CIPRES Science Gateway web portal (http://www.phylo.org/sub_sections/portal/). The alignment and tree are included in Supplementary material 1.

We also used T-BAS 2.1 (Carbone et al. 2019) and the "Place Unknowns" tool to place newly generated ITS sequences onto the Pezizomycotina tree version 2. Two FASTA files of the newly generated ITS sequences of *Spiropes* were uploaded to the T-BAS interface. We selected the "de novo" option for the RAxML placement, with 500 bootstrap replicates.

Results

Taxonomy

Based on morphological evidence, the hyperparasitic fungi collected in Panama and Benin are assigned to the genera *Atractilina* or *Spiropes*. Among these, three species are proposed as new to science, all in the genus *Spiropes*. Four species represent new reports for Benin, and three for Panama. We also present a revision from herbarium material of 17 of the 19 known species

of the genus *Spiropes* and one species of *Atractilina* hyperparasitic on Meliolales. All species synonyms, unless specified, are taken from Deighton and Pirozynski (1972) for *Atractilina parasitica*, and from Ellis (1968) for species of *Spiropes.*

Atractilina Dearn. & Barthol., *Mycologia* 16: 175, 1924.

Atractilina parasitica (G. Winter) Deighton & Piroz., *Mycol. Pap.* 128: 34, 1972. (Fig. 1)
   ≡ *Arthrosporium parasiticum* G. Winter, *Hedwigia* 25: 103, 1886.
   ≡ *Arthrobotryum parasiticum* (G. Winter) Hansf., *Proc. Linn. Soc. Lond.* 155: 64, 1943.
= *Isariopsis penicillata* Ellis & Everh., *Bull. Torrey bot. Club* 22: 438, 1895.
   ≡ *Phaeoisariopsis penicillata* (Ellis & Everh.) S.C. Jong & E.F. Morris, *Mycopath. Mycol. appl.* 34: 271, 1968.
= *Arthrobotryum tecomae* Henn., *Hedwigia* 43: 397, 1904.
= *Arthrobotryum caudatum* Syd. & P. Sydow, *Etudes sur la Flore du Bas et Moyen Congo* 3(1): 22, 1909.
= *Arthrobotryum dieffenbachiae* F. Stevens, *Bot. Gaz.* 65: 237, 1918.
= *Atractilina callicarpae* Dearn. & Barthol., *Mycologia* 16: 175, 1924.
= *Podosporium pallidum* Pat., *Scient. Surv. P. Rico* 8(1) Bot.: 103, 1926.
= *Eriomycopsis bosquieae* Hansf., *Bothalia* 4(2): 466, 1942.
= *Arthrobotryum deightonii* Hansf., *Mycol. Pap.* 15: 218, 1946.
= *Malacaria meliolicola* Syd., *Annls. Mycol.* 28(1/2): 69, 1930. **New synonym proposed in this study.**
= *Paranectria flagellata* Hansf., *Proc. Linn. Soc. London* 153(1): 28, 1941. **New synonym proposed in this study.**
   ≡ *Malacaria flagellata* (Hansf.) Hansf., *Mycol. Pap.* 15: 128, 1946. **New synonym proposed in this study.**

Colonies effuse, rust brown or pale brown, with hyphae that form large, erect, dark synnemata clearly visible under the stereomicroscope, but sometimes only loose unstalked tufts around the tips of the setae of the meliolalean host. **Hyphae** superficial, branched, septate, thin-walled, 1–2.5 μm wide, smooth. **Conidiophores** may form straw-coloured or pale olivaceous synnemata up to 1.5 mm long, 40 μm wide at the basal stalk-like part. Sometimes the synnemata grow around and up the setae of the meliolalean host. Individual conidiophores straight or sometimes flexuous, cylindrical, 2.5–5 μm thick towards the apex, pale olivaceous brown, with denticles. **Conidia** solitary, straight or slightly curved, fusiform, truncate at the base, tapering towards the apex and often terminating in a little bulbous swelling, 1 to mostly 3 septate, thin-walled, variable in size, (17–)30–37(–80) x (3.5–)7–8.5 μm, at first more or less colorless, at maturity becoming pale straw coloured, minutely rough-walled. As seen by SEM, the ornamentation of the surface of the conidia is distinctly reticulated, with thin networks and no ridges.

Specimens examined – On *Meliola* sp. on living leaves of *Opilia celtidifolia* (Opiliaceae), Benin, Campus University of Abomey-Calavi, botanical garden, 6°25'7"N; 2°20'34"E, 24 m a.s.l., 9 February 2022, M. A. Bermúdez-Cova, A. Tabé, D. Dongnima, O.P. Agbani, M. Piepenbring, N.S. Yorou, MB 127 (UNIPAR, M); on *Meliola clerodendricola* on living leaves of *Clerodendrum capitatum* (Lamiaceae), Benin, Abomey-Calavi, Zopah, 6°30'8"N; 2°20'24"E, 37 m a.s.l., 12 February 2022, M. A. Bermúdez-Cova, A. Tabé, D. Dongnima, O.P. Agbani, M. Piepenbring, N.S. Yorou, MB 133; on *Meliola clerodendricola* on living leaves of *Clerodendrum capitatum*, Benin, Allada, Sékou, 6°38'56"N; 2°11'38"E, 48 m a.s.l., 12 February 2022, M. A. Bermúdez-Cova, A. Tabé, D. Dongnima, O.P. Agbani, M. Piepenbring, N.S. Yorou, MB 136 (UNIPAR, M, GenBank accession number: OR804686); on *Meliola* sp. on living leaves of *Pterocarpus santalinoides* (Fabaceae), Benin, Lokoli, border of forest, 7°3'41"N; 2°15'26"E, 22 m a.s.l., 20 February 2022, M. A. Bermúdez-Cova, A. Tabé, D. Dongnima, L. Konetche, M. Piepenbring, R. Hounkarin, MB 160 (M, UNIPAR); on *Meliola* sp. on living leaves of *Coffea arabica* (Rubiaceae), Benin, Attogon, Niaouli, CRA-Sud center, 6°44'24"N; 2°8'25"E, 122 m a.s.l., 28 February 2022, M. A. Bermúdez-Cova, A. Tabé, I. Agonglo, M. Piepenbring, N.S. Yorou, O.P. Agbani, MB 178 (UNIPAR, M, GenBank accession numbers: OR804685 and OR804687); on *Meliola* sp. on living leaves of *Coffea arabica*, Benin, Atlantique, Attogon, Niaouli Forest, 6°44'23"N; 2°8'26"E, 119 m a.s.l., 19 September 2022, A. Krauß, A. Tabé, O. Koukol, N.S. Yorou, AK06H (UNIPAR, M, GenBank accession number: OR804684); on *Meliola* sp. on living leaves of *Clerodendrum capitatum*, Benin, Atlantique, Attogon, Pahou Forest, 6°22'56"N; 2°9'35"E, 13 m a.s.l., 6 October 2022, A. Krauß, A. Tabé, O. Koukol, N.S. Yorou, AK61.

Additional specimens examined – On *Meliola lasiotricha* on leaves of unknown plant host, Puerto Rico, 1926, M.B. Ellis (IMI 130722, type specimen of *Podosporium pallidum*); On *Meliola clerodendri* on leaves of *Clerodendrum cyrtophyllum*, Taiwan, 1938, W. Yamamoto (IMI 31921b, type specimen of *Atractilina parasitica*).

Illustrations – This species was illustrated by Deighton and Pirozynski (1972).

Known hosts and distribution – On colonies of *Amazonia* spp., *Asteridiella* spp., *Irenopsis* spp. and *Meliola* spp. on living leaves of various plants in Congo, Ghana, Guinea, India, Mauritius, Nigeria, Perú, Philippines, Puerto Rico, Sierra Leone, St. Thomé, Taiwan, Tanzania, Uganda, the U.S.A., Venezuela. Only one single collection on *Balladyna* sp. (Balladynaceae,

Dothideomycetes) as a fungal host (Deighton & Pirozynski 1972). *Atractilina parasitica* is reported here for the first time for Benin.

Notes – Only two species of the genus *Atractilina* with hyperparasitic lifestyle are known, namely *A. asterinae* and *A. parasitica* (Deighton and Pirozynski 1972). *Atractilina asterinae* differs from *A. parasitica* by the presence of 3–10 septate, thick-walled conidia.

The specimens of *A. parasitica* collected on leaves of *Coffea arabica* (MB 178, AK4H, AK06H) were found growing together with pseudothecia of *Malacaria meliolicola* Syd. (Tubeufiales, Dothideomycetes). According to Hansford (1941, as *Paranectria flagellata*; 1946), *M. flagellata* is most probably the perfect state of *A. parasitica.* The specimens collected by Hansford were also growing on coffee leaves. The latter, and the fact that the DNA sequences we obtained from *A. parasitica* (GenBank accession numbers: OR804684, OR804686, OR804685 and OR804687) and *M. meliolicola* (GenBank accession numbers: OR805247 and OR805248) clustered together in one single strongly supported clade (Fig. 22), confirm the anamorph-teleomorph connection between both species. For an updated species description of *M. meliolicola*, see Bermúdez-Cova et al. (2023b).
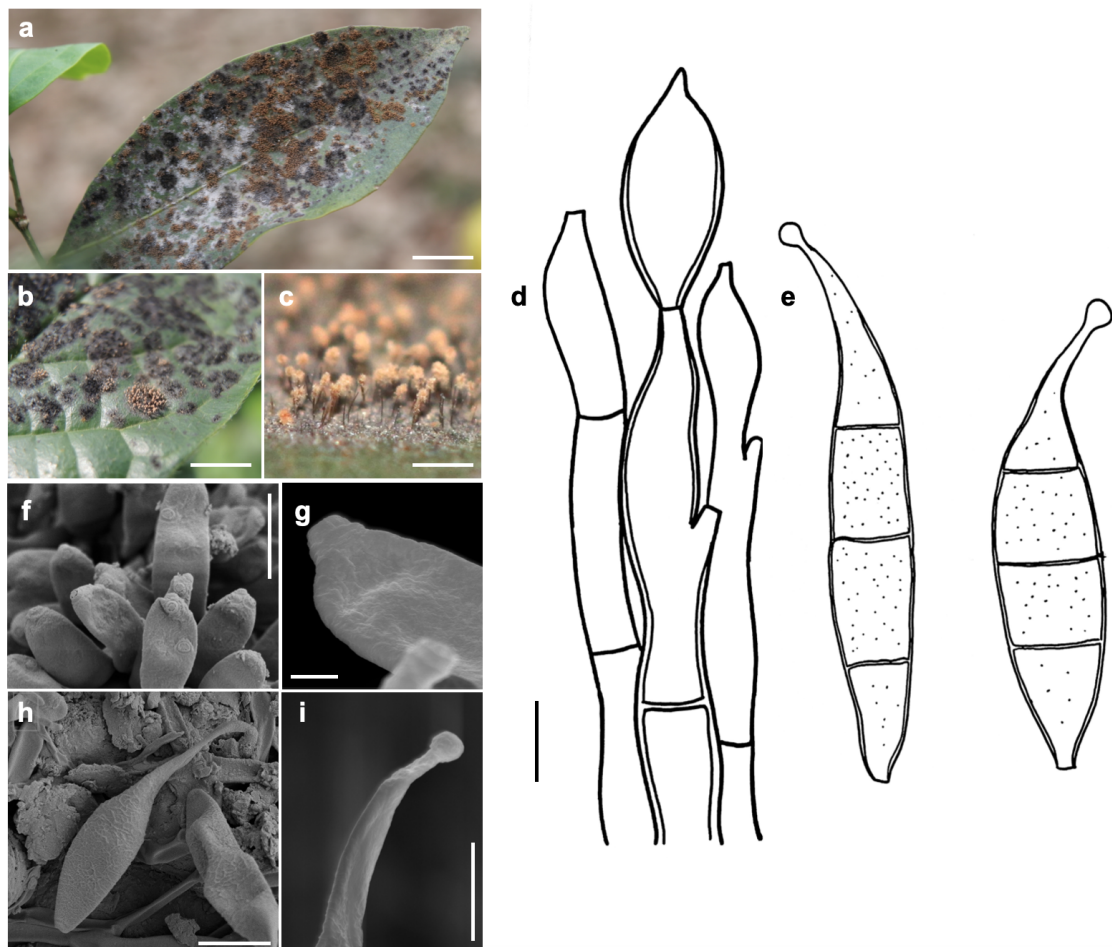
Figure 1 *Atractilina parasitica* **(MB127, MB136). A** Synnemata (gold spots) on colonies of *Meliola* sp. (black spots) on a leaf of *Opilia celtidifolia* **B** Synnemata of (gold spots) on colonies of *Meliola clerondendricola* (black spots) on a leaf of *Clerodendrum capitatum* **C** Synnemata **D** Conidiophores drawn in optical section. The thickness of the wall is indicated only in the drawing in the middle **E** Conidia shown in optical section **F–I** As seen by SEM **F** Conidiophores with denticles **G** A denticle at the tip of a conidiophore **H** Conidium **I** Bulbous swelling at the tip of a conidium. Scale bars: 1.5 mm (**B**); 1 mm (**C**); 5 μm (**D, E, I**); 8 μm (**F**); 1 μm (**G**); 6 μm (**H**).

Spiropes Cif., *Sydowia* 9(1–6): 302, 1955.

Spiropes angylocalycis Berm.-Cova & M. Piepenbr., sp. nov. (Fig. 2)

MycoBank: MB#850990.

Holotype – On *Meliola* sp. on living leaves of *Angylocalyx oligophyllus* (Fabaceae), Benin, Atlantique, Attogon, Niaouli Forest, 6°44'42"N; 2°7'50"E, 69 m a.s.l., 28 February 2022, M.A. Bermúdez, A. Tabé, D. Dongnima, I. Agonglo, O.P. Agbani, M. Piepenbring, N.S. Yorou, MB 167 (M).

Etymology – Named after the genus of the host plant.

Colonies effuse, dark brown to black, velvety to hairy. **Hyphae** superficial, branched, anastomosing, septate, 0.5–2 μm wide, straw-coloured, smooth. **Conidiophores** arising singly, erect or ascending, straight to flexuous, mostly flexuous at the tips, septate, up to 350 μm long, 4–6 μm thick, pale olivaceous brown to brown, with rough surface, with scattered scars mostly in upper parts of the conidiophores. **Conidia** solitary, straight or slightly curved, fusiform to obclavate, 3–septate, (15–)17–25(–30) x 5–6.5 μm, 2–3 μm wide at the base, brown, the cells at each end pale brown, septa darker in color, verrucose. As seen by SEM, the ornamentation of the spores is distinctly reticulated, with thin to thick networks and no ridges.

Known distribution – On colonies of *Meliola* sp. on living leaves of *Angylocalyx oligophyllus* in Benin.

Notes – *Spiropes angylocalycis* is similar to *S. clavatus* by the presence of 3–septate mostly fusiform conidia, with a similar size range (Ellis 1968). However, the conidiophores of *S. clavatus* are synnematous, while they are mononematous in *S. angylocalycis.*
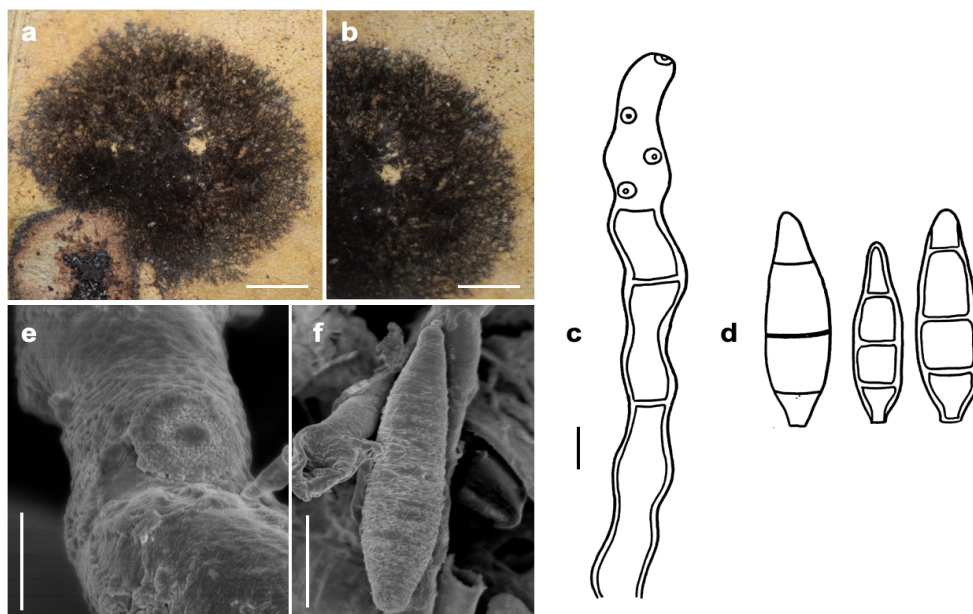
Fig. 2 *Spiropes armatellae* **(MB 167). a–b** Conidiophores growing intermingled with hyphae of *Meliola* sp. on leaves of *Angylocalyx oligophyllus*; **c** Conidiophore with scars; **d** Conidia shown in optical section. The thickness of the wall is shown in the two drawings on the right-hand side; **e–f** As seen by SEM. **e** Part of a conidiophore with scar; **f** Conidium. Scale bars: 0.3 mm (**a**); 0.2 mm (**b**); 5 μm (**c, d**); 2 μm (**e**); 7 μm (**f**).

Spiropes armatellae M.B. Ellis, *Mycol. Pap.* 125: 15, 1971. (Fig. 3)

Type – On *Armatella cinnamomicola* on leaves of *Cinnamomum* sp. (Lauraceae)**,** Sri Lanka, Ceylon, 1971, M.B. Ellis (IMI134405b. The type specimen was not available for loan).

Colonies effuse, dark brown to black, hairy. **Hyphae** superficial, branched, septate, 1–3 μm wide, straw-colored, smooth. **Conidiophores** arising singly, erect or ascending, straight to flexuous, mostly flexuous at their tips, septate, up to 300 μm long, 5–8 μm thick, brown to dark brown, paler towards the apex, with rough surface, with scattered scars in upper parts of the conidiophores. **Conidia** solitary, straight or slightly curved, obclavate to obpyriform, mostly 1–septate, (20–)30–42(–50) x (6–)7–8(–10) μm, 2–3.5 μm wide at the base, brown, paler towards the ends, verrucose when seen by LM and SEM.

Specimen examined – On *Armatella litseae* on leaves of *Daphnidium pulcherrimum* (Lauraceae), India, West Bengal, 1967, M.K. Maity (IMI 136371); on *Armatella cinnamomicola* on leaves of *Cinnamomum* sp., Myanmar, Thaton, 1971, M.M. Thaung, (IMI 161265).

Known hosts and distribution – On colonies of *Armatella* spp. on various plants in India, Myanmar and Sri Lanka (Ellis 1971).

Illustrations – This species was illustrated by Ellis (1971).

Notes – Two known species of *Spiropes* are hyperparasitic on species of the genus *Armatella* (Meliolales, Armatellaceae), namely *S. armatellae* and *S. armatellicola* (Ellis 1971,
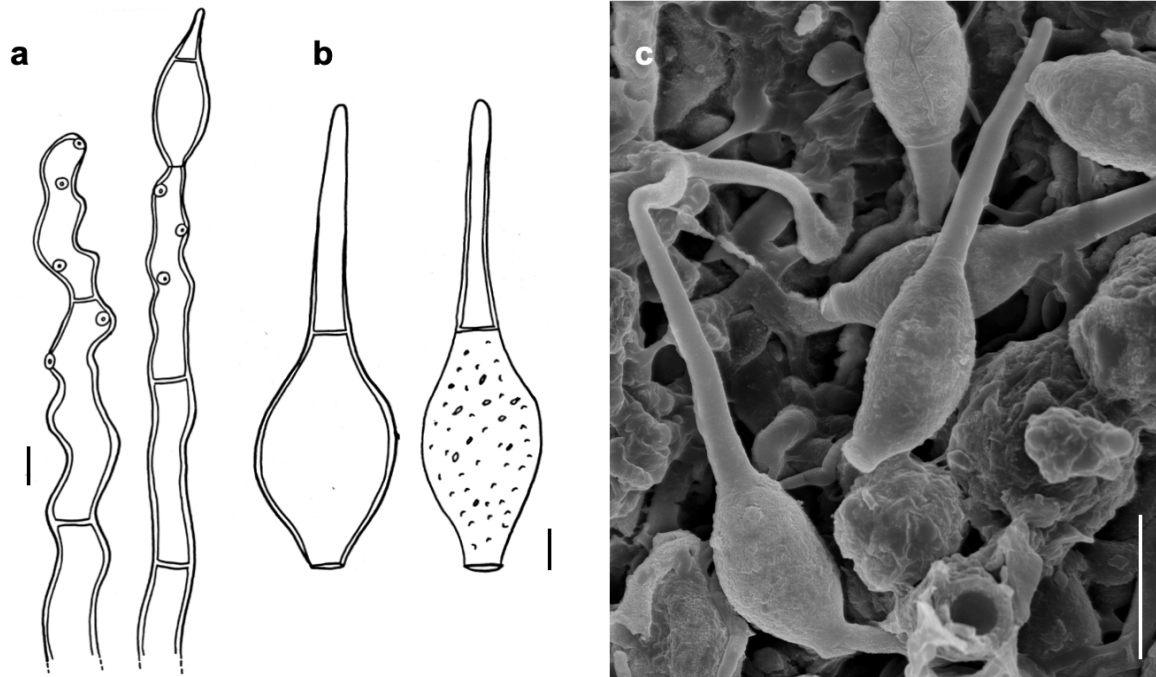
Hosagoudar et al. 2002). According to Hosagoudar et al. (2002), both species are similar, but differ by the ornamentation of the conidia. The conidia of *S. armatellicola* are smooth, while those of *S. armatellae* are distinctly reticulated. However, it is sometimes difficult to observe the surface of the conidia by LM. Therefore, we recommend to analyze the ornamentation of the spores of *S. armatellicola* by SEM. The scars of *S. armatellae* could not be observed by SEM, and it is necessary to collect fresh specimens of this fungus for further morphological analysis.

Fig. 3 *Spiropes armatellae* **(IMI 161265). a** Conidiophores with young conidium; **b–c** Conidia **b** Shown in optical section. The thickness of the wall is indicated only in the drawing on the left-hand side; **c** As seen by SEM. Scale bars: 5 μm (**a**); 2,5 μm (**b**); 10 μm (**c**).

Spiropes armatellicola M.B. Ellis, *Mycol. Pap.* 125: 15, 1971.

Type – On *Armatella* sp. on leaves of *Actinodaphne* sp. (Lauraceae), Banasuran Hills, Wyanad, Kerala, India, April 16, 1999, C.K. Biju (HCIO 43621. The type specimen was not available for loan by HCIO).

Species description – This species was described by Hosagoudar et al. (2002).

Known hosts and distribution – On colonies of *Armatella* sp. on living leaves of *Actinodaphne* sp. in India (Hosagoudar et al. 2002).

Illustrations – This species was illustrated by Hosagoudar et al. (2002).

Notes – This species is only known from the type specimen.

Spiropes capensis (Thüm.) M.B. Ellis, *Mycol. Pap.* 114: 5, 1968. (Fig. 4)

≡ *Cercospora capensis* (Thüm.) Sacc., *Syll. fung.* 4: 469, 1886.
≡ *Helminthosporium capense* (Thüm.) [as '*Helmisporium*'], *Flora*, Regensburg 59: 570, 1876.
≡ *Pleurophragmium capense* (Thüm.) S. Hughes, *Can. J. Bot.* 36: 796, 1958.
= *Helminthosporium carpocrinum* Cif. [as '*Helmisporium*'], *Annls. Mycol.* 36(2/3): 236, 1938.
= *Helminthosporium coffeae* Massee [as '*Helmisporium*'], *Bull. Misc. Inf.,* Kew: 167, 1901.
≡ *Sporhelminthium coffeae* (Massee) Speg., *Physis*, Rev. Soc. Arg. Cienc. Nat. 4(no. 17): 292, 1918.
= *Helminthosporium fici* H.S. Yates [as '*ficuum*'], *Philipp. J. Sci. (Bot.)* 13: 382, 1918.
= *Helminthosporium ficinum* Sacc. [as '*Helmisporium*'], *Atti Accad. Sci. Ven.-Trent.-Istr.*, Sér. 3, 10: 90, 1919.
= *Helminthosporium fumagineum* Sacc. [as '*Helmisporium*'], *Atti Accad. Sci. Ven.-Trent.-Istr.*, Sér. 3, 10: 90, 1919.
= *Helminthosporium filicicola* Henn., *Hedwigia* 44: 71, 1905.
= *Helminthosporium glabroides* F. Stevens [as '*Helmisporium*'], *Bot. Gaz.* 65(3): 240, 1918.
= *Helminthosporium melioloides* Sacc. [as '*Helmisporium*'], *Atti Accad. Sci. Ven.-Trent.-Istr.*, Sér. 3, 10: 89, 1919.
= *Helminthosporium orbiculare* Lév., *Annls. Sci. Nat., Bot.*, Sér. 3, 5: 299, 1846.
= *Helminthosporium philippinum* Sacc. [as '*Helmisporium*'], *Atti Accad. Sci. Ven.-Trent.-Istr.*, Sér. 3, 10: 89, 1919.
= *Helminthosporium subsimile* Sacc., *Boll. Orto bot., Napoli* 6: 23, 1921.
= *Helminthosporium tapurae* Allesch., *Hedwigia* 36(4): 245, 1897.
= *Napicladium portoricense* Speg., *Boln Acad. nac. Cienc. Córdoba* 26(2-4): 363, 1921.
≡ *Helminthosporium portoricense* (Speg.) Cif., *Sydowia* 9(1–6): 298, 1955.
= *Nascimentoa pseudoendogena* Cif. & Bat., *Publicações Inst. Micol. Recife* 44:4, 1956.

Colonies effuse, dark brown to black, hairy (Ellis 1968). **Hyphae** superficial, branched, septate, 2–4 µm wide, pale olive to olivaceous brown, smooth. **Conidiophores** arising singly or in groups, sometimes in large groups of 50–100 conidiophores, terminally or laterally from the hyphae, erect or ascending, straight or flexuous, septate, up to 600 µm long, 5–9 µm thick

along most of their length, brown to dark brown, paler closer to the apex, with terminal and lateral scars. **Conidia** solitary, straight or curved, fusiform to obclavate, truncate at the base, 3–6 (usually 4 or 5) pseudosepta, (33–)50–60(–78) x (5.5–)6–11(–16) µm, 1–4 µm wide at the base, light brown to brown, smooth.

Specimen examined – On *Meliola* sp. on living leaves of *Coffea arabica*, Benin, Atlantique, Attogon, Niaouli Forest, 6°44'23"N; 2°8'26"E, 119 m a.s.l., 19 September 2022, A. Krauß, A. Tabé, O. Koukol, N.S. Yorou, AK06H.

Additional specimens examined – On leaves of *Ficus ulmifolia* (Moraceae), Philippines, Los Baños, 1915, C.F. Baker, 451 (IMI 130940, type of *Helminthosporium fumagineum*); on *Meliola compositarum* on leaves of *Eupatorium portoricense* (Asteraceae)**,** Puerto Rico, Bega Vaja, 1921, no. 1753 (IMI 100331a, type of *Napicladium portoricense*).

Known hosts and distribution – On colonies of *Appendiculella* spp.*, Asteridiella* spp.*, Irenopsis* spp. and *Meliola* spp. on living leaves of various plants in Amboina, Bolivia, Brazil, Cameroon, Congo, Dominican Republic, Ghana, India, Jamaica, Malaya, Peru, Philippines, Puerto Rico, Sabah, Sierra Leone, South Africa, Tanzania, Trinidad, Uganda and Venezuela (Ellis 1968); on *Meliola* sp. on living leaves of *Coffea arabica* in Benin (this study). *Spiropes capensis* is reported here for the first time for Benin.

Illustrations – This species was illustrated by Ellis (1968).

Notes – According to the nomenclatural and taxonomic database Index Fungorum (http://www.IndexFungorum.org), the current name of the *Spiropes capensis* is *Pleurophragmium capense* (Thüm.) S. Hughes. The genus *Pleurophragmium* (*incertae sedis*, Ascomycota) was established by Costantin (1888) and it comprises species with brown to dark brown conidiophores and sympodially proliferating, denticulate conidiogenous cells producing holoblastic, simple, mostly 3–septate, brown to dark brown conidia (Abarca et al. 2007). According to Ellis (1968), the flat double scar is a good taxonomic character to distinguish species of *Spiropes* from *Pleurophragmium*, since in the latter the conidia are borne at the tips of tapered denticles. The morphological analysis of our samples and the type specimens (AK06H, IMI 100331a and IMI 130940) revealed the presence of flat double scars (Fig. 4e) and no denticles. We think that the examined species differs morphologically from species in the genus *Pleurophragmium*, and therefore it should be retained in the genus *Spiropes.*
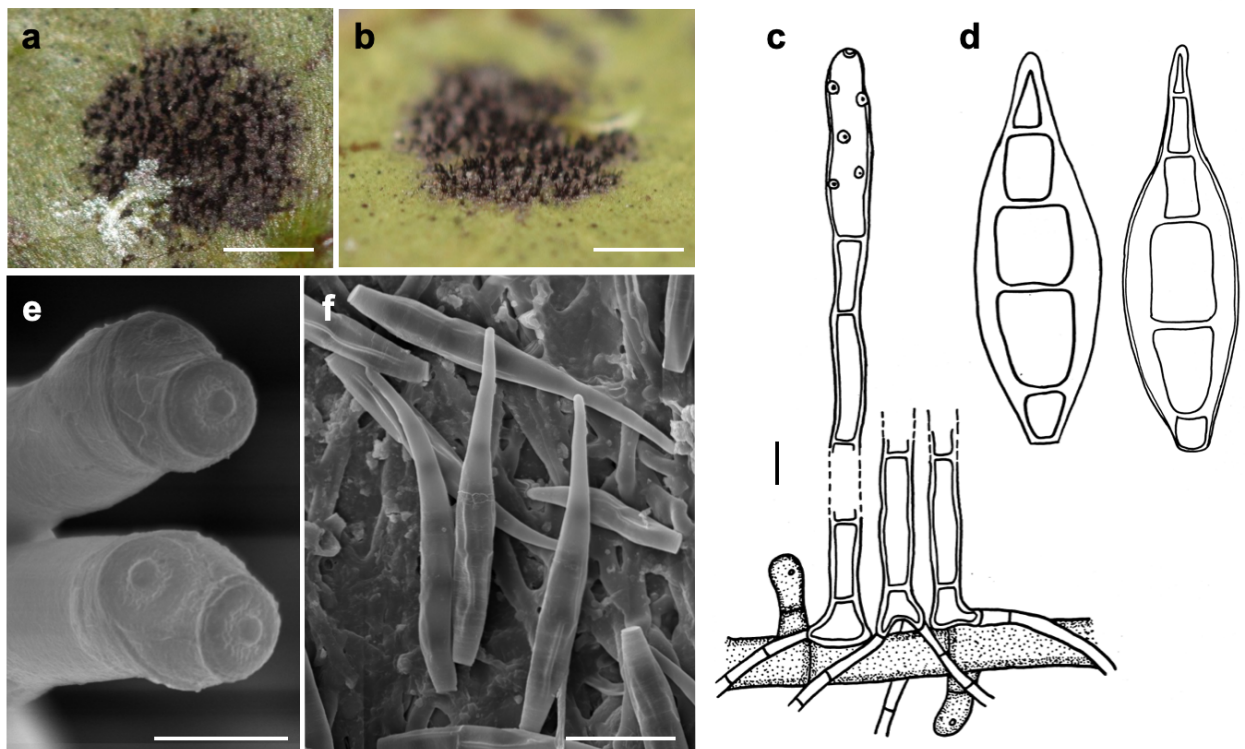
Fig. 4 *Spiropes capensis* **(AK06H). a, b** Groups of conidiophores growing on hyphae of *Meliola* sp.; **c** Conidiophores growing on hyphae of *Meliola* sp. shown in optical section; **d** Conidia shown in optical section. The thickness of the outer wall layer is indicated only in the drawing on the right-hand side; **e–f** As seen by SEM. **e** Conidiophores with scars; **f** Conidia. Scale bars: 1 mm (**a, b**); 8.5 μm (**c**); 5 μm (**d**); 5 μm (**e**); 20 μm (**f**).

Spiropes caribensis Hol.-Jech., *Česká Mykol.* 38(2): 113, 1984. (Fig. 5)

Colonies effuse, dark brown to black, velvety to hairy. **Hyphae** superficial, branched, septate, 1.5–3.5 μm wide, pale olivaceous brown, smooth. **Conidiophores** arising singly, erect or ascending, straight or flexuous, septate, up to 240 μm long, 4–8 μm thick, pale brown to brown, smooth, with few scars. **Conidia** solitary, straight or slightly curved, obclavate, central cells barrel-shaped, 3-septate, (30–)36–48(–41.5) x (7.5–)9.5–11.5 μm, 4.5–6 μm wide at the truncate base, the central cells pale brown, the cells at the ends paler and almost hyaline, smooth.

Specimen examined – On *Meliola* sp. on leaves of an unknown palm-tree, Cuba, Isla de La Juventud (= Isla de Pinos), Los Indios, south-west of La Cañada, 1981, V. Holubová-Jechová (PRM 831531, holotype).

Known hosts and distribution – On *Meliola* sp. on living leaves of an unidentified palm tree in Cuba (Holubová-Jechová and Mercado Sierra 1984).

Illustrations – This species was illustrated by Holubová-Jechová and Mercado Sierra (1984).

Notes – *Spiropes caribensis* is similar to *S. helleri*, but differs from the latter by paler conidia, with wider truncate base (*S. helleri* has conidia with a truncate base 3–4 μm wide), and shorter conidiophores (up to 600 μm long in *S. helleri*; Holubová-Jechová & Mercado Sierra, 1984). As seen by SEM, conidia of *S. caribensis* are smooth (Fig. 5b), while conidia of *S. helleri* are distinctly reticulated (Fig. 13e). The scars could not be observed by SEM, and it is therefore necessary to collect fresh specimens of this fungus for further morphological analyses. *S. caribensis* is only known from the type specimen.
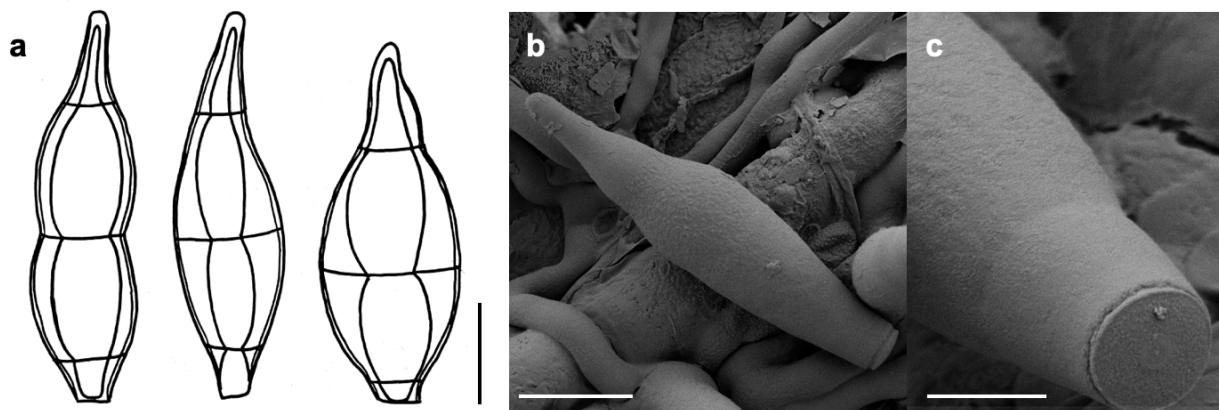

Fig. 5 *Spiropes caribensis* (PRM 8311531). a Conidia shown in optical section; **b–c** As seen by SEM. **b** Conidium; **c** Basis of a conidium with a flat scar. Scale bars: 10 μm (**a**); 9 μm (**b**); 4 μm (**c**).

Spiropes carpolobiae Berm.-Cova & M. Piepenbr., sp. nov. (Fig. 6)

MycoBank: MB#850987.

Holotype – On *Meliola* cf. *carpolobiae* on living leaves of *Carpolobia lutea* (Polygalaceae), Benin, Atlantique, Attogon, Niaouli Forest, 6°44'41"N; 2°7'52"E, 68 m a.s.l., 28 February 2022, M.A. Bermúdez, A. Tabé, D. Dongnima, I. Agonglo, O.P. Agbani, M. Piepenbring, N.S. Yorou, MB 166 (M).

Etymology – Named after the genus of the host plant.

Colonies effuse, dark brown to black, velvety to hairy. **Hyphae** superficial, branched, anastomosing, septate, 1–2 μm wide, straw-coloured, smooth. **Conidiophores** arising singly, erect or ascending, straight to flexuous, septate, up to 250 μm long, 2–5 μm thick, sometimes thicker at the apex, brown, not smooth, with scattered scars mostly in the upper parts of the conidiophores. **Conidia** solitary, straight or slightly curved, ovate to slightly fusiform, 3–septate, (12.5–)13–16(–19) x 5–7 μm, 2–2.5 μm wide at the base, brown, the cells at each end

pale brown, septa darker, surface verrucose. As seen by SEM, the ornamentation of the conidia is distinctly reticulated, with thin to thick networks that can form ridges.

Known distribution – On colonies of *Meliola* cf. *carpolobiae* on living leaves of *Carpolobia lutea* in Benin.

Notes – S. carpolobiae is the only known species of *Spiropes* with ovate to slightly fusiform conidia.
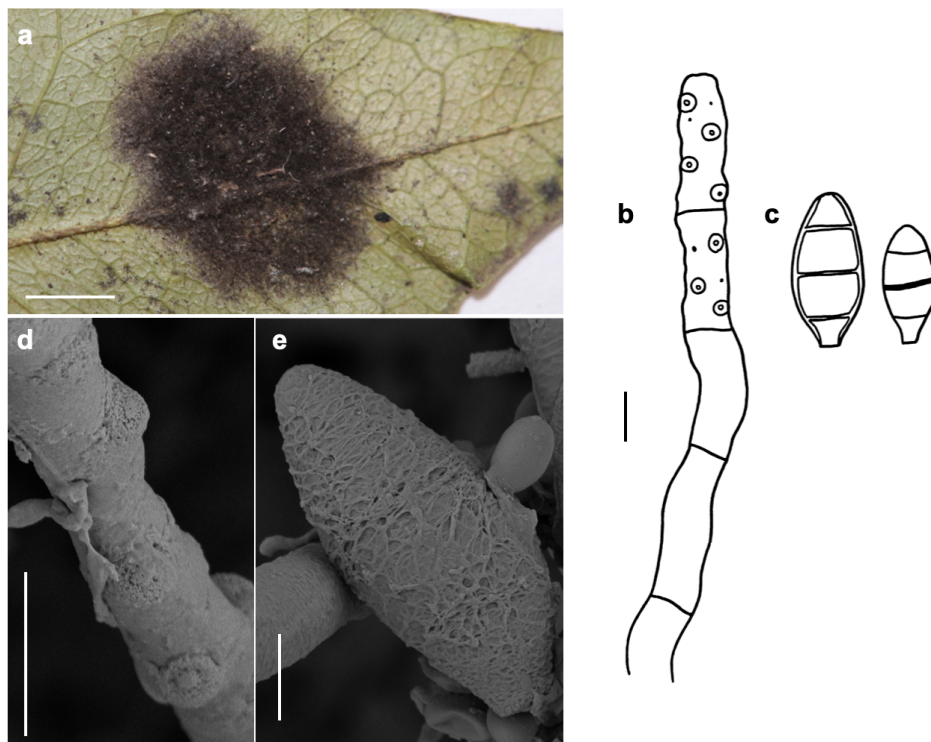
Fig. 6 *Spiropes carpolobiae* **(MB 166). a** Conidiophores growing intermingled with hyphae of *Meliola* sp. on a leaf of *Carpolobia lutea*; **b** Conidiophore with scars; **c** Conidia shown in optical section. The thickness of the wall is shown in the left-hand drawing; **d–e** As seen by SEM. **d** Conidiophore with scar; **e** Conidium. Scale bars: 0.3 mm (**a**); 5 μm (**b, c**); 5 μm (**d**); 3 μm (**e**).

Spiropes clavatus (Ellis & Martin) M.B. Ellis, *Mycol. Pap.* 114: 25, 1968. (Fig. 7)

  ≡ *Isariopsis clavata* Ellis & Martin, *Am. Nat.* 18: 188, 1884.
  ≡ *Arthrobotryum clavatum* (Ellis & Martin) Höhn, *Sber. Akad. Wiss. Wien,* Math.-naturw. Kl., Abt. 1, 125: 120, 1916.
  ≡ *Bitunicostilbe clavata* (Ellis & Martin) M. Morelet, *Bull. Soc. Sci. nat. Arch. Toulon et du Var* 7: 195, 1971.
= *Podosporium chlorophaeum* Speg., *An. Mus. nac. Hist. nat. B. Aires* 20: 450, 1910.
= *Arthrobotryum noz-moscatae* Bat. & J. Silva, *Anais IV Congr. Soc. bot. Brasil*: 144, 1953.

Colonies effuse, brown to dark brown or black. **Hyphae** superficial, branched, anastomosing, septate, 1–3 µm wide, pale olivaceous brown. **Conidiophores** tightly packed to form dark brown to blackish synnemata up to 700 µm long, 20–40 µm thick, often splaying out to a width of up to 110 µm at the apex. Individual hyphae straight or flexous, cylindrical, 1–3 µm thick near the base, 4–7 µm thick near the apex, dark brown, paler towards the apex, verrucose, with numerous conidial scars. **Conidia** solitary, fusiform to obclavate, mostly 3–, rarely 1–, 2– or 4–septate, (13–)18–25(–33) x (4–)5–7(–8) µm, tapering to about 1–1.5 µm at the apex and at the base, pale brown to brown, the cells at each end paler, wrinkled. As seen by SEM, the ornamentation of the spores is distinctly reticulated, with thin to thick networks and no ridges.

Specimens examined – On *Meliola panici* on leaves of *Panicum glutinosa*, Puerto Rico, El Alto de la Bandera, 1913, F.L. Stevens & W.E. Hess, n°4368 (IMI 130764); on *Meliola* sp. on leaves of *Raphia monbuttorum*, Uganda, 1915, R. Dümmer, IMI 102772; on *Meliola thouiniae* on leaves of an unknown plant, Brasil, São Paulo, 1940, A.R. Campos (IMI 130975, type of *Arthrobotryum noz-moscatae*).

Illustrations – This species was illustrated by Ellis (1968).

Known hosts and distribution – On colonies of Meliolales on living leaves of various plants in Argentina, Brazil, Ghana, Malaysia, Puerto Rico, Sierra Leone, Trinidad and Uganda (Ellis 1968).

Notes – In the nomenclatural and taxonomic database Index Fungorum (http://www.IndexFungorum.org), the current name of the *Spiropes clavatus* is *Bitunicostilbe clavata* (Ellis & Martin) M. Morelet. The genus *Bitunicostilbe* (*incertae sedis*, Ascomycota) was proposed by Morelet (1971) to accommodate two species, namely *B. clavata* and *B. linderae*, that were previously cited in other genera. Although the publication by Morelet was not available for this study, the morphological analysis of the herbarium specimens (IMI 130764, 130975) revealed that the features of these specimens are consistent with the description of *Spiropes clavatus* by Ellis (1968). The species has typical characteristics of the genus *Spiropes*, such as flat double scars (Fig. 7c) and therefore, it should be classified in this genus. De Beer et al. (2013) analyzed the type and additional specimens of *B. linderae* (as *Graphium linderae*), and concluded that this species should be also classified in the genus *Spiropes*.
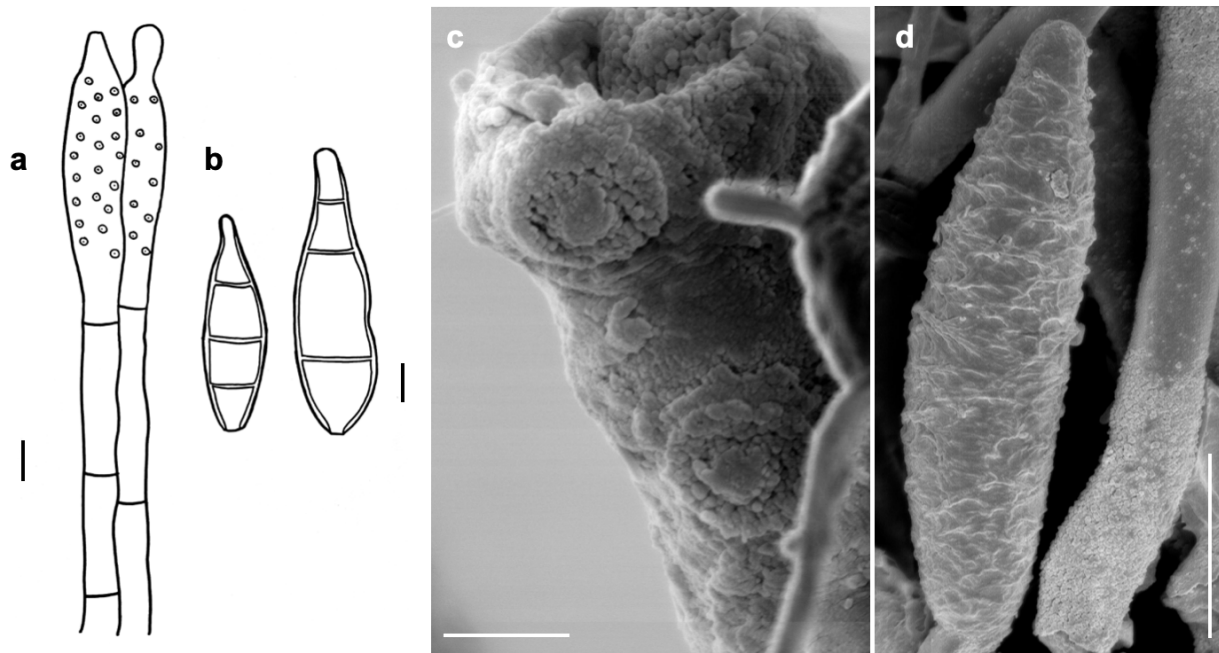
Fig. 7 *Spiropes clavatus* **(IMI 102772). a** Conidiophores with scars; **b** Conidia shown in optical section; **c–d** As seen by SEM. **c** Conidiophore with scars; **d** Conidium. Scale bars: 5 μm (**a**); 2.5 μm (**b**); 1 μm (**c**); 5 μm (**d**).

Spiropes croissantiformis Berm.-Cova & M. Piepenbr., sp. nov. (Fig. 8)

MycoBank: MB#850984.

Holotype – On *Meliola* cf. *xylopiae* on living leaves of *Xylopia frutescens*, Panama, Chiriquí Province, Cochea, Cochea river trail, 8° 32' 37" N 82° 23' 03" W, 181 m a.s.l., 26 February 2020, M.A. Bermúdez, A. Sanjur, A. Villarreal, MB110 (UCH).

Etymology – Named after the shape of the conidia.

Colonies effuse, dark brown to black, with tightly packed hyphae that form erect, dark synnemata clearly visible under the stereomicroscope. Hyphae superficial, branched, septate, 1–2 μm wide, straw-coloured, smooth. Conidiophores tightly packed to form dark brown to blackish synnemata up to 400 μm high, spreading out at the apex, up to 80 μm diam. Individual hyphae mostly straight, cylindrical, 3–5 μm thick, with numerous small scars, brown, paler towards the apex, rough. Conidia straight or curved, mostly crescent shaped, sometimes fusiform, mostly 3(–5)–septate, (14–)20–24(–33) x (3.5–)5–6.5 μm, with two golden brown middle cells and paler cells at each. As seen by SEM, the ornamentation of the spores is distinctly reticulated, with thin to thick networks and no ridges.

Known distribution – On colonies of *Meliola* cf. *xylopiae* on living leaves of *Xylopia frutescens* (Annonaceae) in Panama.

Notes – Spiropes xylopiae is a synnematous hyperparasitic species of *Spiropes* with the shortest synnemata (up to 400 µm), when compared to other synnematous species, such as *S. melanoplaca* with synnemata that can reach up to 1.5 mm and *S. penicillium* with synnemata up to 700 µm high. In addition to this, the new species has crescent-shaped conidia, a feature that is not present in any other known species of the genus.
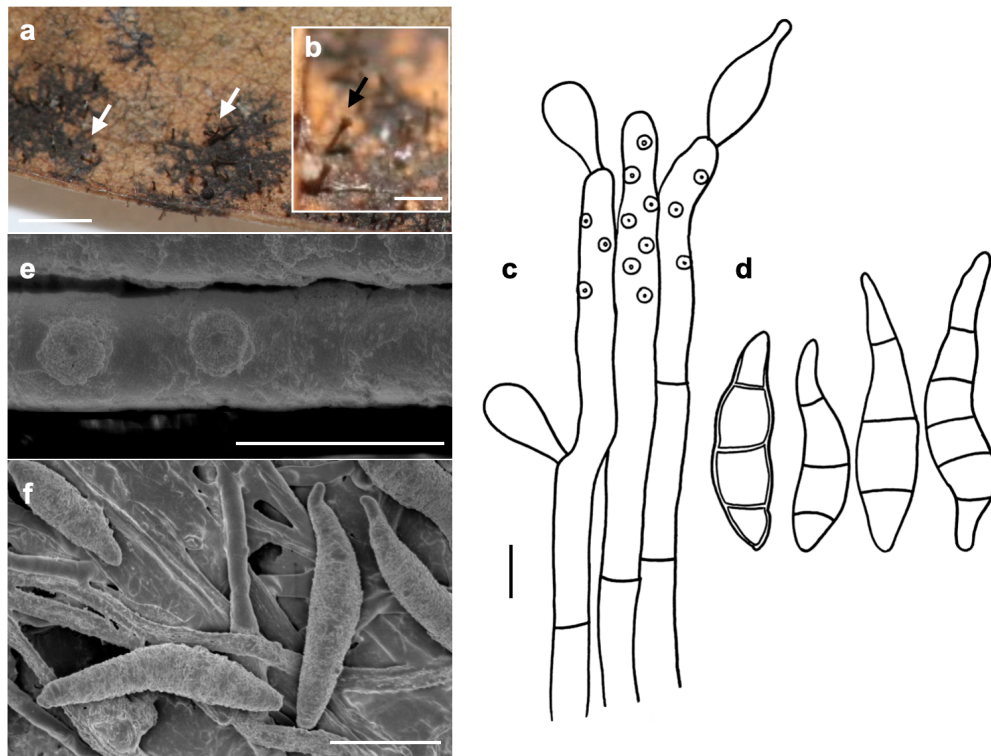
Fig. 8 *Spiropes croissantiformis* **(MB 110). a** Synnemata (indicated by white arrows) growing on colonies of *Meliola* cf. *xylopiae*; **b** Synnema (indicated by a black arrow); **c** Conidiophores with scars and young conidia, shown in optical section; **d** Conidia shown in optical section. The thickness of the wall is only shown for the first spore from the left; **e–f** As seen by SEM. **e** Part of a conidiophore with scars; **f** Conidia. Scale bars: 160 µm (**a**); 400 µm (**b**); 5 µm (**c, d**); 5 µm (**e**); 10 µm (**f**).

Spiropes deightonii M.B. Ellis, *Mycol. Pap.* 114: 18, 1968. (Fig. 9)

Colonies effuse, olive to olivaceous brown, velvety or hairy. **Hyphae** superficial, branched, septate, 0.5–2 µm wide, pale olive to olivaceous brown, smooth. **Conidiophores** arising singly or in groups terminally or laterally from the hyphae, erect or ascending, straight or flexous, septate, up to 400 µm long, 2-4 µm thick along most of their length, swollen towards the apex, 5–8 µm thick, brown, reticulate as seen by SEM, with scattered cylindrical scars. **Conidia** solitary, straight or slightly curved, obovate to clavate, truncate at their base, 3–septate, (10–

)12–14(–15) x (5–)6–8 μm, 1.5–2 μm wide at the base, the cells at each end of a conidium subhyaline or pale brown, intermediate cells brown, ornamented. As seen by SEM, the ornamentation of the spores is distinctly reticulated, with thin to thick networks that can form ridges.

Specimen examined – On *Meliola borneensis* on *Uvaria chamae*, Sierra Leone, 1951, F.C. Deighton, (IMI 48956a, type of *S. deightonii*).

Illustrations – This species was illustrated by Ellis (1968).

Known hosts and distribution – On colonies of *Meliola borneensis* on living leaves of *Uvaria chamae* (Annonaceae) in Sierra Leone (Ellis 1968).

Notes – *Spiropes deightonii* and *Spiropes intricatus* are the only known species of the genus that present conidiophores that swell in the areas where conidia are formed (Figs. 9, 14; Ellis 1968). *Spiropes intricatus* differs from *S. deightonii* by the presence of larger conidia (16–23 μm long) that are more oblong-ellipsoid (Ellis 1968), rather than obovate or clavate. *S. deightonii* is only known from the type specimen.

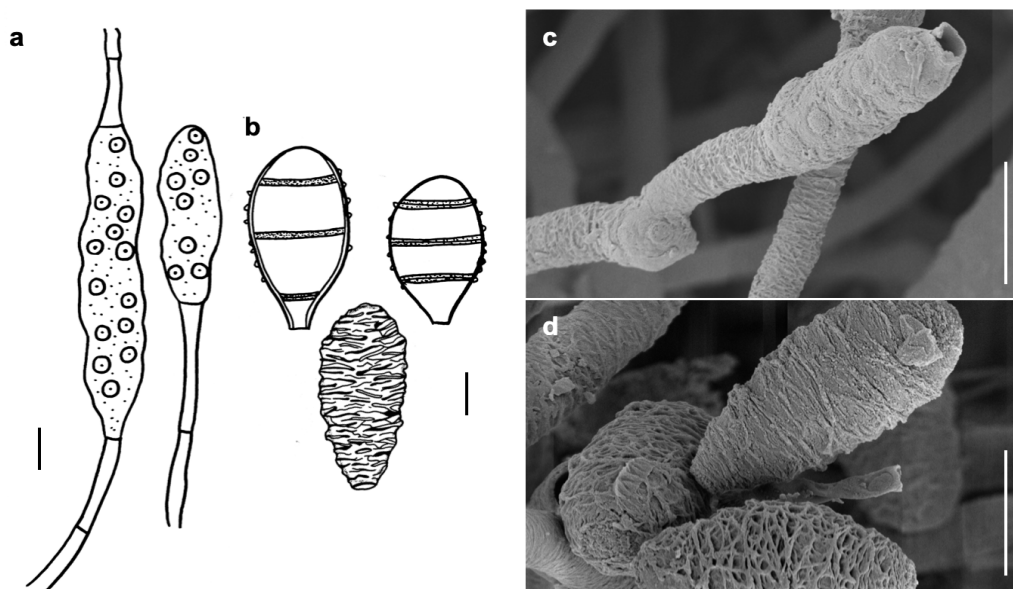**Fig. 9** *Spiropes deightonii* (IMI48956a). **a** Conidiophores; **b** Conidia, as seen by LM (two upper spores; the thickness of the wall is indicated only in the drawing on the left-hand side) and by SEM (bottom spore); **c–d** As seen by SEM. **c** Conidiophore; **d** Conidia. Scale bars: 5 μm (**a, b**); 8 μm (**c**); 5 μm (**d**).

Spiropes dorycarpus (Mont.) M.B. Ellis, *Mycol. Pap.* 114: 27, 1968. (Fig. 10)

≡ *Helminthosporium dorycarpum* Mont., *Annls Sci. nat.*, 2 Sér., 17: 120, 1842.

≡ *Pleurophragmium dorycarpum* (Mont.) Hughes, *Can. J. Bot.* 36: 797, 1958.

= *Helminthosporium orbiculare* Lév., *Annls Sci. nat.*, 3 Sér., 5: 299, 1846.

= *Napicladium myrtacearum* Speg., *An. Soc. cient. Argent.* 26: 71, 1888.

≡ *Sporhelminthium myrtacearum* (Speg.) Speg., *Physis* 4(17): 292, 1918.

= *Helminthosporium conspicuum* McAlpine, *Proc. Linn. Soc. N.S.W.* 22: 40, 1897.

= *Podosporium densum* Pat., *J. Bot. Paris* 11: 373, 1897.

= *Helminthosporium asterinoides* Sacc. & P. Syd., *apud Saccardo, Rc. Congr. Bot. Palermo, May 1902*: 58, 1902.

≡ *Sporhelminthium asterinoides* (Sacc. & Syd.) Speg., *Physis* 4(17): 292, 1918.

= *Helminthosporium melastomacearum* F. Stevens, *Bot. Gaz.* 65: 242, 1918.

= *Helminthosporium panici* F. Stevens, *Bot. Gaz.* 65: 242, 1918.

= *Helminthosporium parathesicola* [as 'parathesicolum'] F. Stevens, *Bot. Gaz.* 65: 242, 1918.

Colonies effuse, brown to dark brown, hairy. **Hyphae** superficial, branched, septate, 1–3 µm wide, straw-coloured, pale brown, smooth. **Conidiophores** arising singly or in groups, terminally or laterally from the hyphae, erect or ascending, straight or flexous, septate, up to 700 µm long, 3–7 µm thick, straw-coloured, pale brown to brown, with scattered cylindrical scars towards the apex. **Conidia** solitary, straight or slightly curved, variable in shape, but mostly obclavate to fusiform, truncate at the base, mostly 3–septate, but sometimes with 4 to 5 septa, (16–)20–35(–40) x (4.5–)5–7 µm, straw-colored to pale brown, middle cells slightly darker, wrinkled or verrucose. As seen by SEM, the ornamentation of the spores is distinctly reticulated, with thin to thick networks and no ridges.

Specimen examined – On *Meliola* sp. on living leaves of *Coffea arabica*, Benin, Atlantique, Attogon, Niaouli Forest, 6°44'23"N; 2°8'26"E, 119 m a.s.l., 19 September 2022, A. Krauß, A. Tabé, O. Koukol, N.S. Yorou, AK06H.

Additional specimens examined – On *Eugenia pungens*, Brasil, Guarapí, 1883, B. Balansa, 3939, (IMI 100322, type of *Napicladium myrtacearum*); on *Meliola* sp. on leaves of an unknown plant, Cuba, R. de la Sagra (IMI 10002, type of *Helminthosporium dorycarpum*).

Illustrations – This species was illustrated by Ellis (1968).

Known hosts and distribution – On colonies of *Appendiculella* spp., *Asteridiella* spp., *Clypeolella* spp., *Irenopsis* spp., *Meliola* spp. and *Schiffnerula* spp., on living leaves of various plants in Australia, Brazil, Chile, Congo, Cuba, Dominican Republic, Ghana, Guyana, India, Malaysia, Nigeria, Puerto Rico, Sierra Leone, South Africa, Taiwan, Tanzania and Uganda (Ellis 1968). *Spiropes dorycarpus* is reported here for the first time for Benin.

Notes – Spiropes dorycarpus is similar to *S. effusus* and *S. helleri* by the presence of non-synnematous conidiophores and conidia mostly with 3 true septa. However, conidia of *S. effusus* are narrower (3–5 µm) than those of *S. helleri* (7–13 µm).
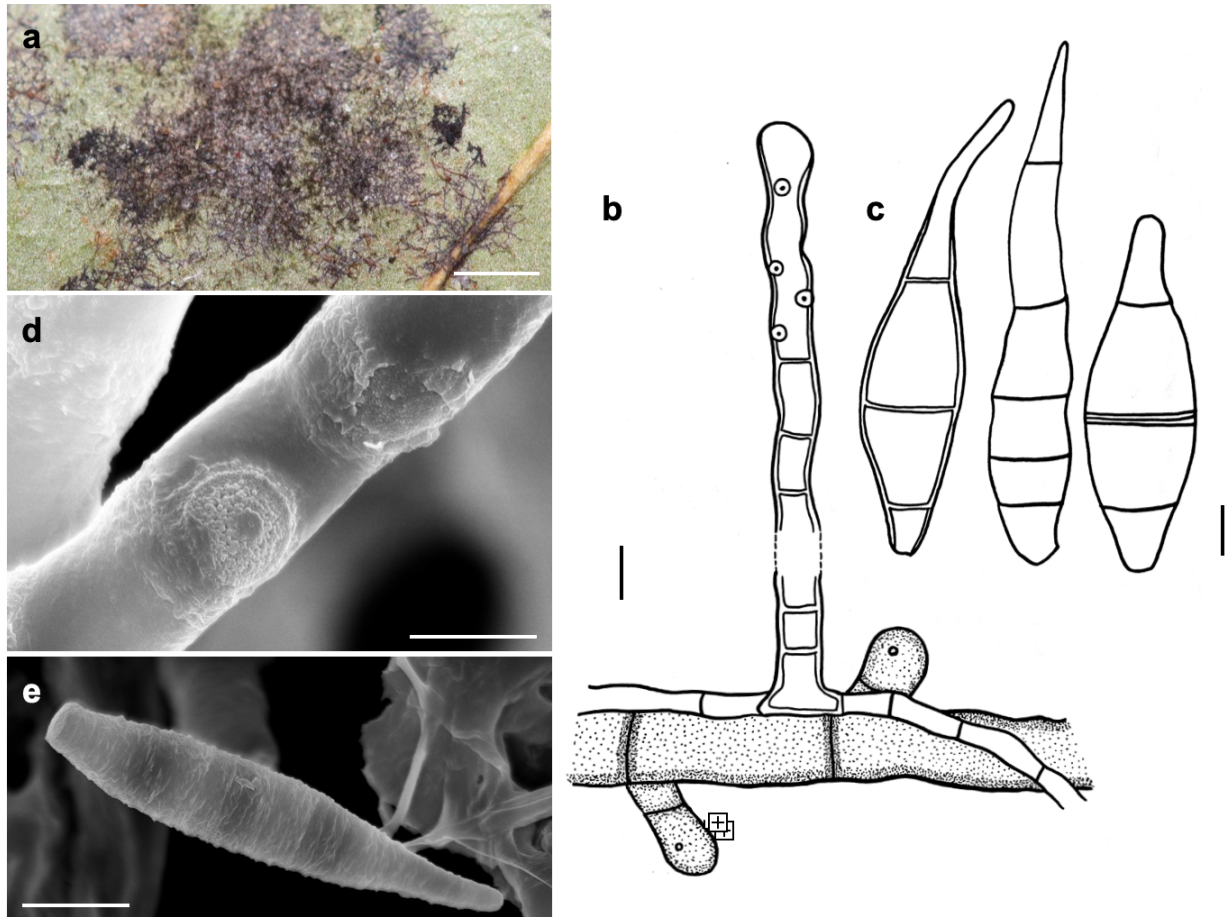
Fig. 10 *Spiropes dorycarpus* (AK06H). a Superficial hyphae growing on a colony of *Meliola* sp. on a leaf of *Coffea arabica*; **b–c** In optical section. **b** Conidiophore growing on a hypha of *Meliola* sp.; **c** Conidia. The thickness of the wall is indicated only in the drawing on the left-hand side; **d–e** As seen by SEM. **d** Conidiophore with a scar; **e** Conidium. Scale bars: 1 mm (**a**); 5 µm (**b**); 3.5 µm (**c**); 3 µm (**d**); 7 µm (**e**).

Spiropes effusus (Pat.) M.B. Ellis, *Mycol. Pap.* 114: 10, 1968. (Fig. 11)

≡ *Podosporium effusum* Pat., *Scient. Surv. P. Rico* 8(1): 103, 1926.
= *Helminthosporium dorycarpum* var. *amazoniae* Hughes [as 'Helmisporium'], *Mycol. Pap.* 50: 24, 1953.
≡ *Pleurophragmium dorycarpum* var. *amazoniae* (S. Hughes) S. Hughes, *Can. J. Bot.* 36: 797, 1958.

Colonies effuse, olive to brown, hairy. **Hyphae** superficial, branched, septate, 1–2 μm wide, yellowish, olive or pale brown, smooth. **Conidiophores** arising singly or in groups, as terminal and lateral branches on the hyphae, erect, straight or flexous, septate, up to 300 μm long, 3–4 μm thick, slightly reticulated when seen by SEM, with few or many small conidial scars towards the apex. **Conidia** solitary, narrowly obclavate to fusiform, truncate at the base, mostly 3(–5)–septate, (15–)20–36 x (3–)3.8–4.5(–5) μm, pale brown, the central cells slightly darker, verruculose. As seen by SEM, the ornamentation of the spores is distinctly reticulated, with thin networks and no ridges.

Specimen examined – On meliolalean fungus on leaves of *Piper* sp., Puerto Rico, Río Piedras, 1926, Heller, 142 (IMI 130721, type of *Podosporium effusum*); on *Amazonia psychotriae* on leaves of *Psychotria warneckei*, Ghana, Togoland, 1938, F.C. Deighton M1617B (IMI 9996a).

Illustrations – This species was illustrated by Ellis (1968).

Known hosts and distribution – On colonies of Meliolales, especially *Amazonia* spp., on living leaves of various plants in Ghana, Puerto Rico, Sierra Leone and Venezuela. One record on *Asterina* sp. (Asterinales, Ascomycota) in Uganda (Ellis 1968).

Notes – Spiropes effusus has conidia similar in size to those of *S. dorycarpus*. However, conidia of *S. dorycarpus* are wider (5–7 μm) than in *S. effusus*.
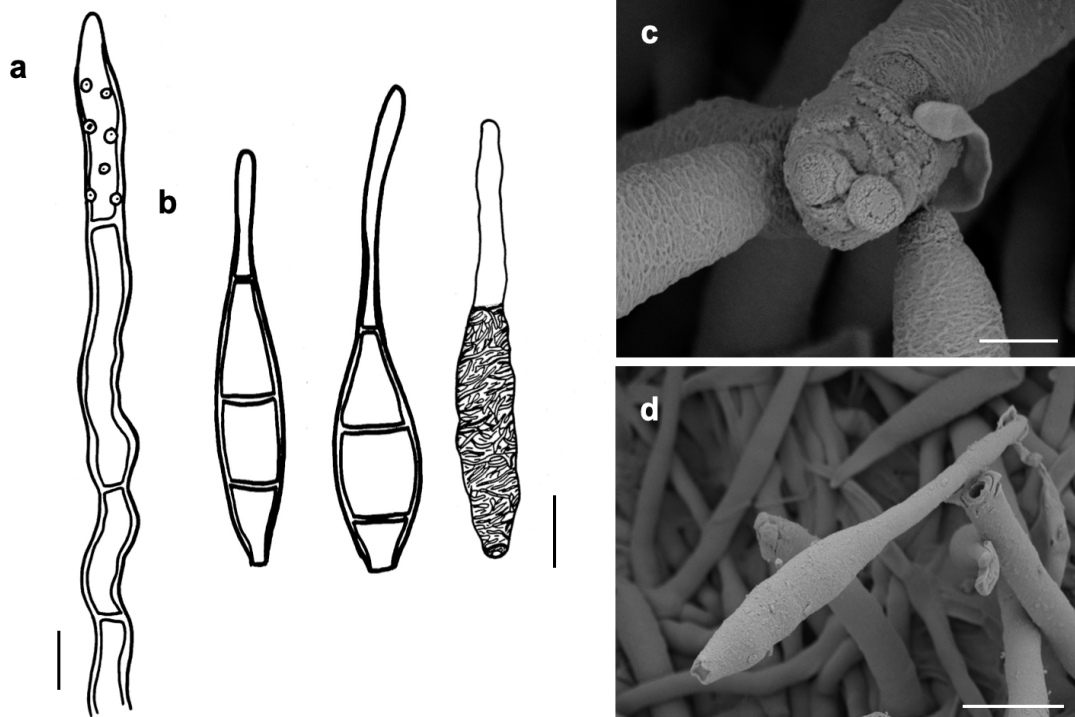
Fig. 11 *Spiropes effusus* **(IMI 130721). a** Conidiophore shown in optical section; **b** Conidia. The first two drawings show spores in optical section. The right-hand drawing shows a conidium as seen by SEM; **c–d** As seen by SEM. **c** Conidiophore with scars and conidia; **d** Conidium. Scale bars: 5 μm (**a**); 8 μm (**b**); 2 μm (**c**); 8 μm (**d**).

Spiropes fumosus (Ellis & Martin) M.B. Ellis, *Mycol. Pap.* 114: 20, 1968.

≡ *Helminthosporium fumosum* Ellis & Martin, *Am. Nat.* 18: 70, 1884.
≡ *Brachysporium fumosum* (Ellis & Martin) Sacc., *Syll. Fung.* 4: 428, 1886.

Type – On *Meliola* sp. on leaves of *Persea palustris* (Lauraceae), Florida, U.S.A, 1883, G. Martin (NY 830274. The type specimen was not available for loan by NY).

Species description – This species was described by Ellis (1968).

Known hosts and distribution – On colonies of *Meliola* sp. on living leaves of *Persea palustris* in the U.S.A. (Ellis 1968).

Specimen examined – On Meliolales on living leaves of *Persea palustris*, U.S.A, Florida, Cove Springs, 1890, G. Martin, (IMI 16307).

Illustrations – This species was illustrated by Ellis (1968).

Notes – The specimen IMI 16307 was analyzed, but no fungal cells were seen.

Spiropes guareicola (F. Stevens) Cif., *Sydowia* 9(1–6): 302, 1955. (Fig. 12)

≡ *Helminthosporium guareicola* F. Stevens [as '*Helmisporium guareicolum*'], *Bot. Gaz.* 65(3): 241, 1918.
≡ *Pleurophragmium guareicola* (F. Stevens) S. Hughes, *Can. J. Bot.* 36: 797, 1958.
= *Cladosporium elegans* var. *singaporense* Sacc., *Bull. Orto Bot. Regia Univ. Napoli* 6: 60, 1921.
= *Helminthosporium flagellatum* H.S. Yates [as '*Helmisporium*'], *Philipp. J. Sci. (Bot.)* 13: 383, 1918.
= *Helminthosporium spirotrichum* Sacc. [as '*Helmisporium*'], *Boll. Orto bot.* 6: 61, 1921.

Colonies effuse, dark brown to black, hairy. **Hyphae** superficial, branched, septate, 2–4 μm wide, pale olivaceous brown, smooth. **Conidiophores** arising singly or in groups, as lateral branches on the hyphae, erect, sterile lower part straight or flexuous, upper fertile part in zigzag shape, septate, up to 400 μm long, 6–9 μm thick, brown to dark brown, paler towards the apex, more or less smooth, with numerous well-defined, dark conidial scars. **Conidia** solitary, broadly fusiform, truncate at the base, with 3 to 5 pseudosepta, (25–)35–52(–60) x (7–)8–10(–

13) µm, 3.5–5 µm wide at the base, pale to dark brown or olivaceous brown, smooth as seen by SEM.

Specimen examined – On leaves of *Cyrtophyllum fragrans* (Gentianaceae)*, Singapore, 1921, Baker (IMI 49160, type of *Helminthosporium spirotrichum*); on *Meliola* sp. on leaves of *Daniellia thurifera* (Fabaceae), Sierra Leone, 1936, F.C. Deightonii M1267 (IMI 10010).

Illustrations – This species was illustrated by Ellis (1968).

Known hosts and distribution – On colonies of *Asteridiella* spp.*, Irenopsis* spp. and *Meliola* spp. on living leaves of various plants in Bougainville islands, Ghana, India, Indonesia, Malaysia, Philippines, Puerto Rico, Sabah, Sierra Leone, Solomon Islands and Uganda (Ellis 1968).

Notes – *Spiropes guareicola* is the type species of the genus *Spiropes*, and it differs from other species of the genus by the presence of zigzag-shaped conidiophores in the fertile upper parts (Ellis 1968). *S. guareicola* presents smooth conidia, a feature that is only evident by SEM.
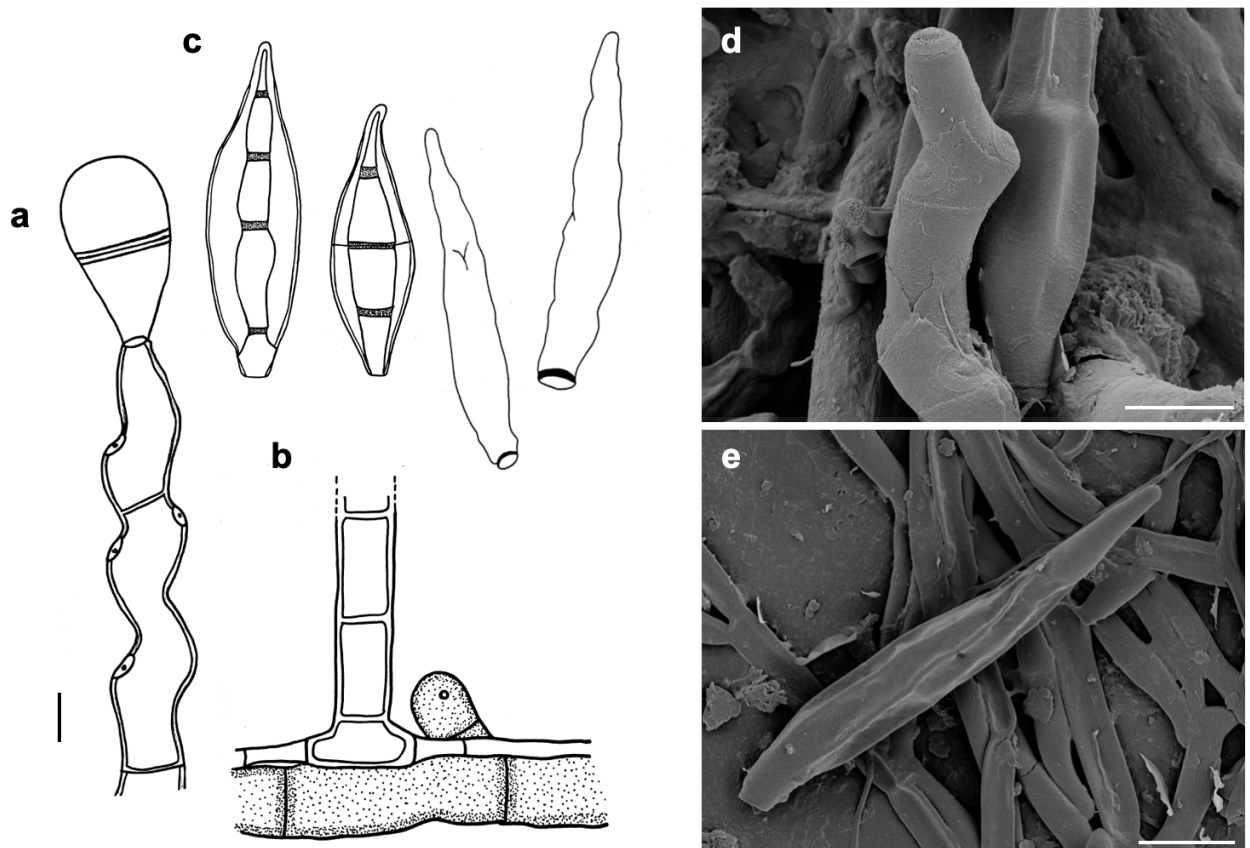
Fig. 12 *Spiropes guareicola* (IMI 10010). **a** Conidiophore with scars and a young conidium shown in optical section; **b** Base of a conidiophore growing on a hypha of *Meliola* sp. shown in optical section; **c** Conidia shown in optical section (two drawings on the left-hand side) and as seen by SEM (two drawings on the right-hand side); **d–e** As seen by SEM. **d** Zigzag-shaped conidiophore with scars; **e** Conidium. Scale bars: 5 μm (**a–c**); 8 μm (**d**); 10 μm (**e**).

Spiropes helleri (F. Stevens) M.B. Ellis, *Mycol. Pap.* 114: 14, 1968. (Fig. 13)

≡ *Helminthosporium helleri* F. Stevens [as '*Helmisporium*'], *Bot. Gaz.* 65(3): 242, 1918.
= *Helminthosporium leucosykes* H.S. Yates [as '*Helmisporium leucosykeae*'], *Philipp. J. Sci., C, Bot.* 13(6): 382, 1918.
= *Helminthosporium maculosum* Sacc. [as '*Helmisporium*'], *Atti Accad. Sci. Ven.-Trent.-Istr.* 10: 91, 1919 [1917].
≡ *Pleurophragmium maculosum* (Sacc.) S. Hughes, *Can. J. Bot.* 36: 797, 1958.

Colonies effused, dark brown to black, hairy. **Hyphae** superficial, branched, septate, 1–3 μm wide, straw coloured or pale brown, smooth. **Conidiophores** arising singly as terminal or lateral branches on the hyphae, erect, straight or flexuous, septate, up to 600 μm long, 5–8 μm wide, brown to dark brown, paler towards the apex, smooth, with scattered conidial scars. **Conidia** solitary, obclavate, frequently rostrate, 3(–4)–septate, (26–)36–43(–50) x (6–)7–10(– 13) μm, 3–4 μm wide at the truncate base, pale brown to brown, verruculose. As seen by SEM, the ornamentation of the spores is clearly reticulated, with thin networks and no ridges.

Specimens examined – On *Meliola* sp. on leaves of *Cupania guatemalensis* (Sapindaceae), Panama, Chiriquí Province, Botanical Garden of the Autonomous University of Chiriquí (UNACHI), 8°25'55"N; 82°27'03"W, 34 m a.s.l., 11 February 2020, M. A. Bermúdez-Cova, A. Sanjur MB92; on *Meliola* sp. on living leaves of *Pterocarpus santalinoides* (Fabaceae), Benin, Atlantique, Attogon, Niaouli Forest, 6°44'40"N; 2°7'53"E, 72 m a.s.l., 20 September 2022, A. Krauß, A. Tabé, O. Koukol, N.S. Yorou, AK15.

Additional specimens examined – On Meliolales on living leaves of an undetermined plant, Gold Coast Colony, Banau, 1949, S.J. Hughes 1141 (IMI44564); on *Meliola* sp. on leaves of *Myrcia deflexa*, Puerto Rico, El Alto de la Bandera, F.L. Stevens 8268 (IMI9991, type of *Helminthosporium helleri*).

Illustrations – This species was illustrated by Ellis (1968).

Known hosts and distribution – On colonies of *Asteridiella* spp.*, Irenopsis* spp. and *Meliola* spp. on living leaves of various plants in Ghana, Malaysia, New Caledonia, Philippines, Puerto

Rico, Sabah, Sierra Leone and Uganda (Ellis 1968). *Spiropes helleri* is reported here for the first time for Benin and for mainland America (Panama).

Notes – *Spiropes helleri* is similar to *S. effusus*, *S. dorycarpus* and *S. leonensis* by the presence of obclavate to sometimes fusiform conidia, but differs from the first two by wider conidia (3.8–4.5 µm in *S. effusus* and 5–7 µm in *S. dorycarpus*), and from the last one by narrower ones (10–11µm).
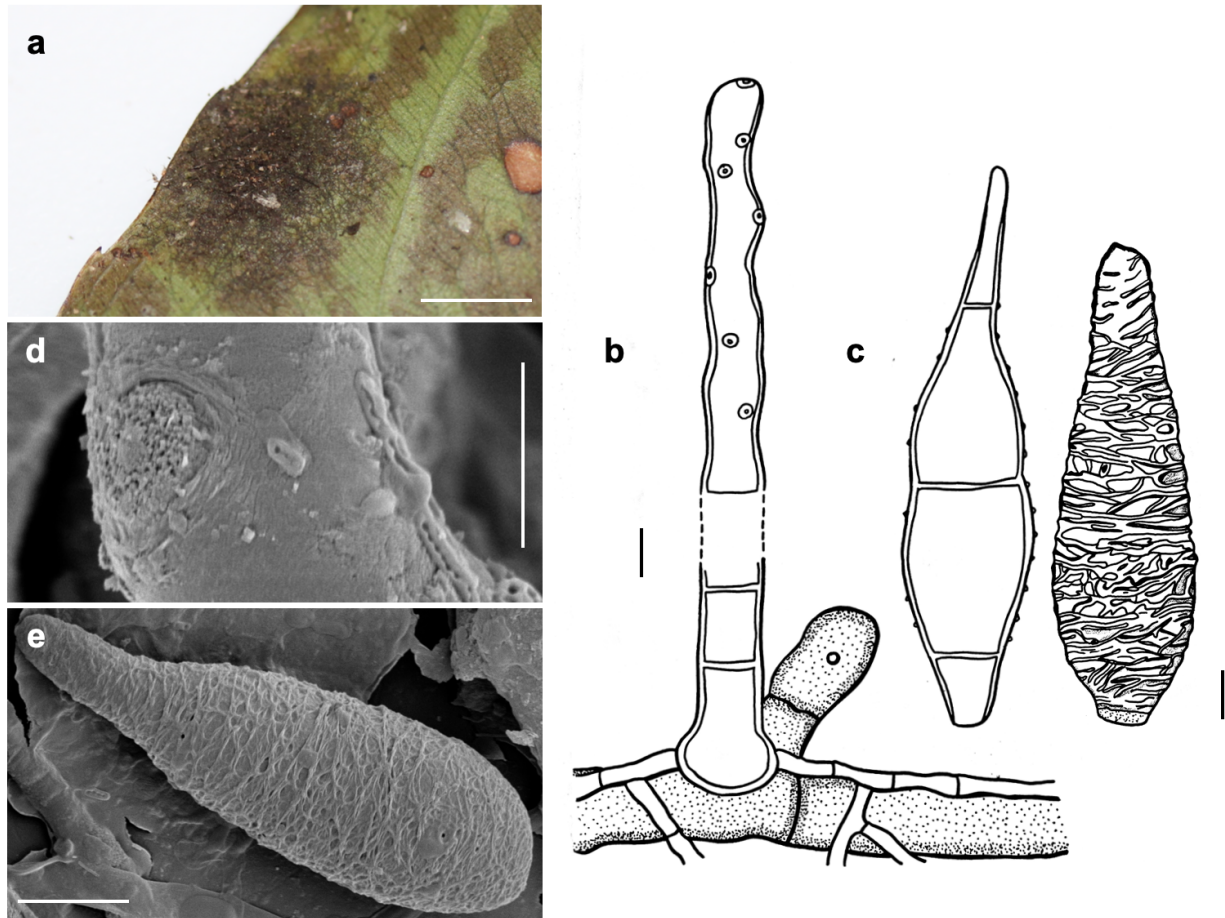
Fig. 13 *Spiropes helleri* **(IMI130940). a** Superficial hyphae growing on a colony of *Meliola* sp. on a leaf of *Cupania guatemalensis*; **b** Conidiophore growing on a hypha of *Meliola* sp. shown in optical section; **c** Conidia shown in optical section (drawing on the left-hand side) and as seen by SEM (drawing on the right-hand side); **d**–**e** As seen by SEM. **d** Part of a conidiophore with a scar; **e** Conidium. Scale bars: 1 mm (**a**); 5 µm (**b**); 6 µm (**c**); 4 µm (**b**); 5 µm (**c**).

Spiropes intricatus (Sacc.) M.B. Ellis, *Mycol. Pap.* 114: 9, 1968. (Fig. 14)

≡ *Brachysporium intricatum* Sacc., *Atti Accad. scient. Veneto-trent.-istriana*, Ser. 3, 10: 88, 1919.
= *Spiropes pirozynskii* M.B. Ellis, *Mycol. Pap.* 114: 19, 1968. **New synonym proposed in this study.**

Colonies effuse, straw-coloured, olive or olivaceous brown, velvety or hairy. **Hyphae** superficial, branched, anastomosing, septate, 1–2 μm wide, pale olivaceous brown, smooth. **Conidiophores** arising singly or in groups, terminally or laterally from the hyphae, erect or ascending, straight or flexuous, septate, up to 900 μm long, 2-5 μm thick along most of their length, swollen to 4–9 μm towards the apex and in intercalary parts that produce conidia, pale olivaceous brown to brown, reticulate as seen by SEM, with scattered cylindrical scars. **Conidia** solitary, straight or slightly curved, oblong-ellipsoid, or obovate to clavate, truncate at the base, mostly 3–septate, (13–)16–23(–25) x (4.5–)6–8 μm, 1.5–3 μm wide at the base, the cells at each end of a conidium pale brown, intermediate cells brown, ornamented. As seen by SEM, the ornamentation of the spores is distinctly reticulated, with thin to thick networks that can form ridges.

Specimens examined – On *Irenopsis* sp. on *Lindackeria bukobensis* (Achariaceae), Tanzania, Kigoma, 1964, K.A. Pirozynski M418 b&c (IMI 106645b-c, type of *Spiropes pirozinskii*); on leaves of *Camellia drupifera* (Theaceae), Nepal, Kathmandu, Godawari, 1986, U. Budathoki KU294 (IMI323287).

Illustrations – This species was illustrated by Ellis (1968).

Known hosts and distribution – On colonies of Meliolales on living leaves of various plants in Ghana, Philippines and Tanzania (Ellis 1968).

Notes – *Spiropes intricatus* and *S. deightonii* are the only known species of the genus that present conidiophores that swell in the areas where conidia are formed (Figs. 9, 14; Ellis 1968). *Spiropes deightonii* differs from *S. intricatus* by the presence of smaller conidia (12–14 μm long) that are more obovate or clavate rather than oblong-ellipsoid. The type specimen of *S. pirozynskii* (IMI 106645b-c) is morphologically similar to *S. intricatus*. Both species present oblong-ellipsoid conidia with a similar size range (Fig. 15). Therefore, we propose *S. pirozinskii* as a synonym of *S. intricatus*.
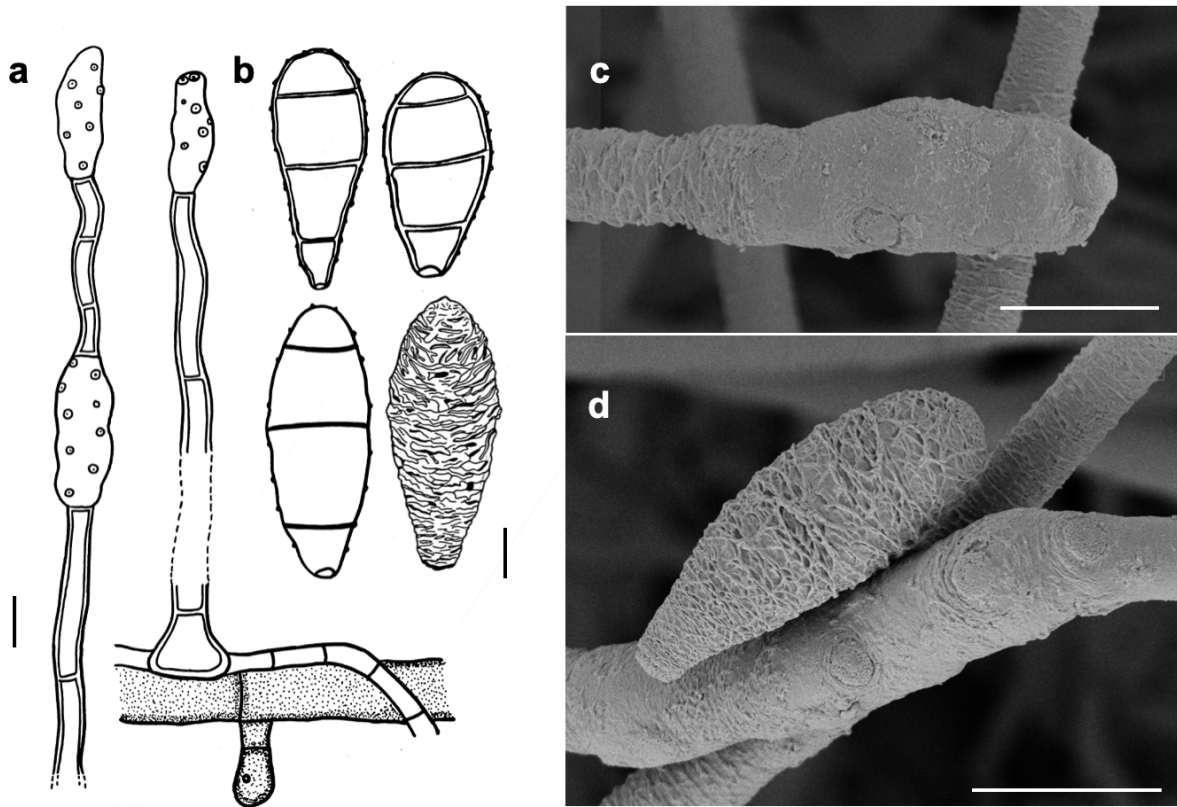
Fig. 14 *Spiropes intricatus* (**IMI 106645b-c**). **a** Conidiophores, growing on a hypha of *Irenopsis* sp., shown in optical section; **b** Conidia shown in optical section (the thickness of the wall is indicated only in the drawings on the upper row), and as seen by SEM (second row right); **c–d** As seen by SEM. **c** Conidiophore with scars; **d** Conidium. Scale bars: 5 μm (**a**); 3 μm (**b**); 7 μm (**c**); 8 μm (**c**).
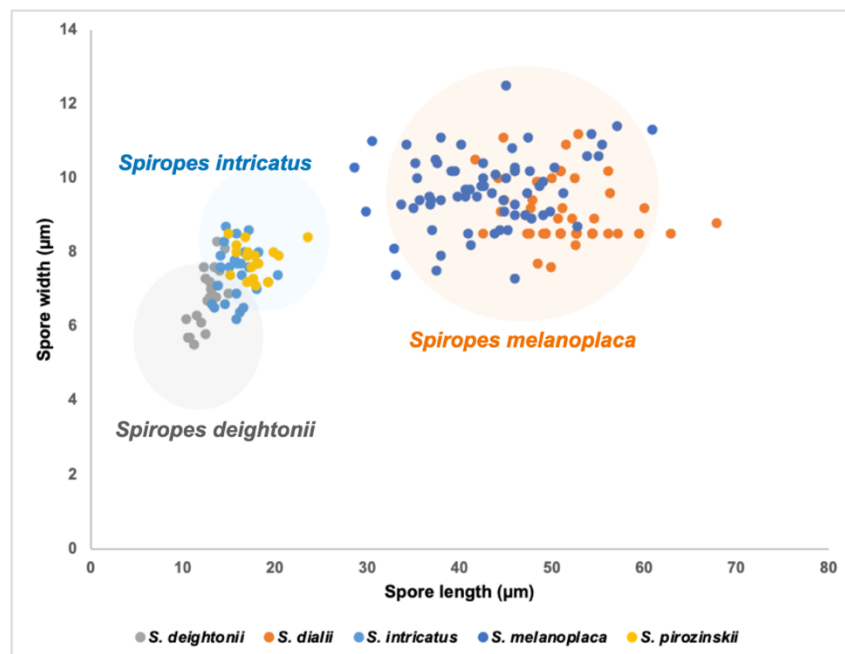
Fig. 15 Scatter plot of spore size (width and length) of Species of *Spiropes*.

Spiropes japonicus (Henn.) M.B. Ellis, *Mycol. Pap.* 114: 22, 1968. (Fig. 16)

≡ *Podosporium japonicum* Henn., *Bot. Jb.* 29: 152, 1900.
= *Helminthosporium insigne* Gaillard ex Sacc. [as '*Helmisporium*'], *Atti Accad. Sci. Ven.-Trent.-Istr.* 10: 89, 1917.

Colonies effuse, amphigenous, sometimes dense, dark brown to black, with tightly packed hyphae that form large, erect, dark synnemata clearly visible under the stereomicroscope. **Hyphae** superficial, branched, septate, 1–4 µm wide, pale olivaceous brown, smooth. **Conidiophores** tightly packed to form dark brown to blackish synnemata up to 1 mm high, spreading out at the apex and upper half of the synnemata; conidiophores individually flexuous or straight, thick-walled, septate, 6–8 µm thick, brown to dark brown at the base, paler towards the apex, smooth, with scattered cylindrical scars. **Conidia** solitary, fusiform to obclavate, with 4(–6) pseudosepta, (50–)67–80 x (7–)8–14 µm, 2–3 µm wide at the apex, 3–5 µm at the truncate base, pale brown to brown, striate.

Specimens examined – On *Meliola* sp. on living leaves of Asteraceae, Panama, Chiriquí Province, Boquerón District, Chuspa Hydroelectric, 8°32'20"N; 82°36'21"W, 281 m a.s.l., 6 March 2020, M. A. Bermúdez-Cova, A. Sanjur, S. Samaniego, MB 120; on *Meliola* sp. on living leaves of Fabaceae, Panama, Chiriquí Province, Bugaba District, area around Gariché River, 8°38'38.1"N; 82°41'19.6"W, 566 m a.s.l., 8 March 2020, M. A. Bermúdez-Cova, A. Sanjur, A. Villarreal, MB 123.

Additional specimens examined – On *Irenina entebbeensis* on *Alchornea hirtella* (Euphorbiaceae), Sierra Leone, 1939, Makump, M1774 (IMI 38813); on *Asteridiella aucubae* on *Aucuba japonica* (Garryaceae), Japan, Ise, 1899, P. Hennings (IMI 130973, type of *Podosporium japonicum*).

Illustrations – This species was illustrated by Ellis (1968).

Known hosts and distribution – On colonies of Meliolales on living leaves of various plants in the Cook Islands, Japan, Malaysia, Papua New Guinea and Sierra Leone (Ellis 1968). *Spiropes japonicus* is reported here for the first time for Panama.

Notes – Spiropes japonicus is the only known synnematous species of *Spiropes* that produces conidia with 4 to 6 pseudosepta, as well as synnemata that splay out at the apex and upper half (Ellis, 1968).
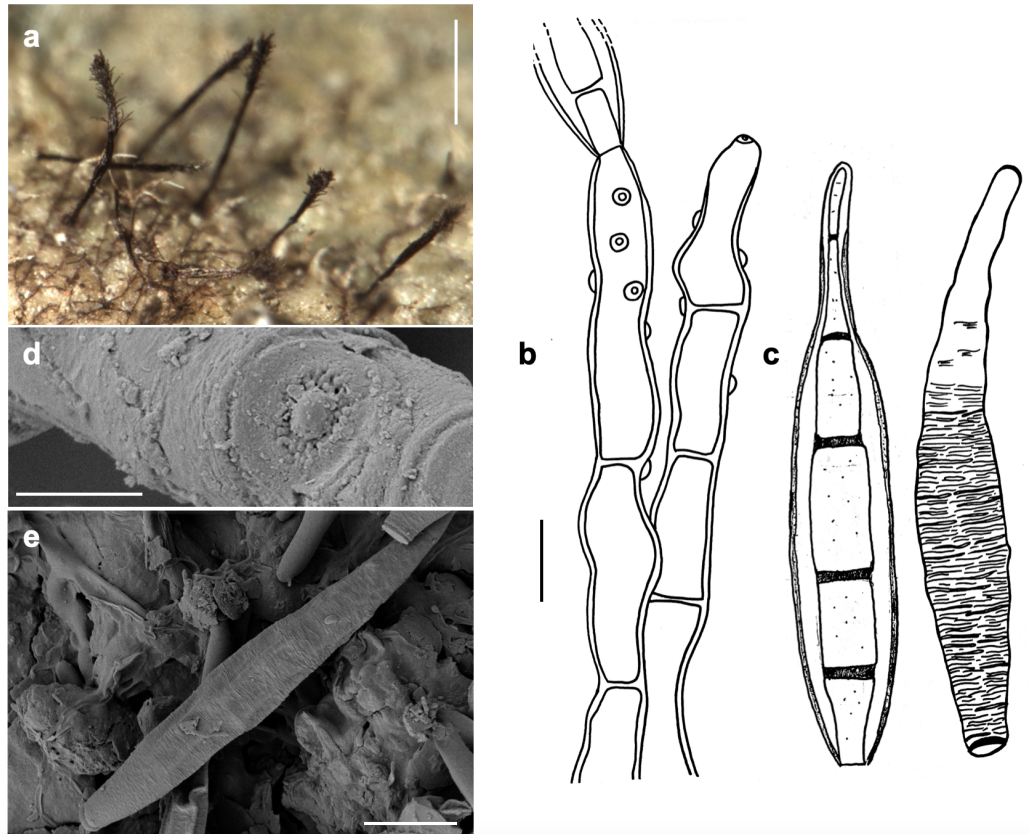
Fig. 16 *Spiropes japonicus* (MB120, 123). a Synnemata growing on a colony of *Meliola* sp.; **b** Conidiophores with scars and a young conidium, shown in optical section; **c** A conidium shown in optical section (drawing on the left) and as seen by SEM (drawing on the right); **d–e** As seen by SEM. **d** Conidiophore with a scar; **e** Conidium. Scale bars: 1 mm (**a**); 10 μm (**b, c**); 3 μm (**d**); 9 μm (**d**).

Spiropes leonensis M.B. Ellis, *Mycol. Pap.* 114: 15, 1968. (Fig. 17)

Colonies effuse, gray to dark blackish brown, hairy. **Hyphae** superficial, branched, septate, 2–6 μm wide, pale brown, smooth. **Conidiophores** arising singly, as terminal and lateral branches on the hyphae, erect, straight or flexuous, septate, up to 700 μm long, 8–12 μm thick, sometimes swollen to 16–17 μm at the base, dark brown to dark blackish brown, paler towards the apex, smooth, with scattered conidial scars. **Conidia** solitary, obclavate, rostrate, 3(–4)–septate, (38–)40–54(–63) x (8–)10–11(–13) μm, 4–6 μm wide at the truncate base, pale brown to brown, verruculose. As seen by SEM, the ornamentation of the spores is distinctly reticulated, with thin networks and no ridges. It was not possible to see the scars by SEM.

Specimen examined – On *Meliola garciniae* on leaves of *Pentadesma butyracea* (Clusiaceae), Sierra Leone, Rokupr, 1951, F.C. Deighton M3920 (IMI 46589b, holotype); on *Meliola garciniae* on *Pentadesma butyracea*, Sierra Leone, near Rokupr, 1939, F.C. Deighton (IMI 9992a, type of *Spiropes leonensis*).

Illustrations – This species was illustrated by Ellis (1968).

Known hosts and distribution – On colonies of *Meliola garciniae* on living leaves of *Pentadesma butyracea* (Clusiaceae) in Sierra Leone (Ellis 1968).

Notes – *Spiropes leonensis* is similar to *S. helleri* by the presence of rostrate, obclavate, 3–septate conidia (Ellis 1968). However, conidia in *S. helleri* are smaller (36–43 µm).
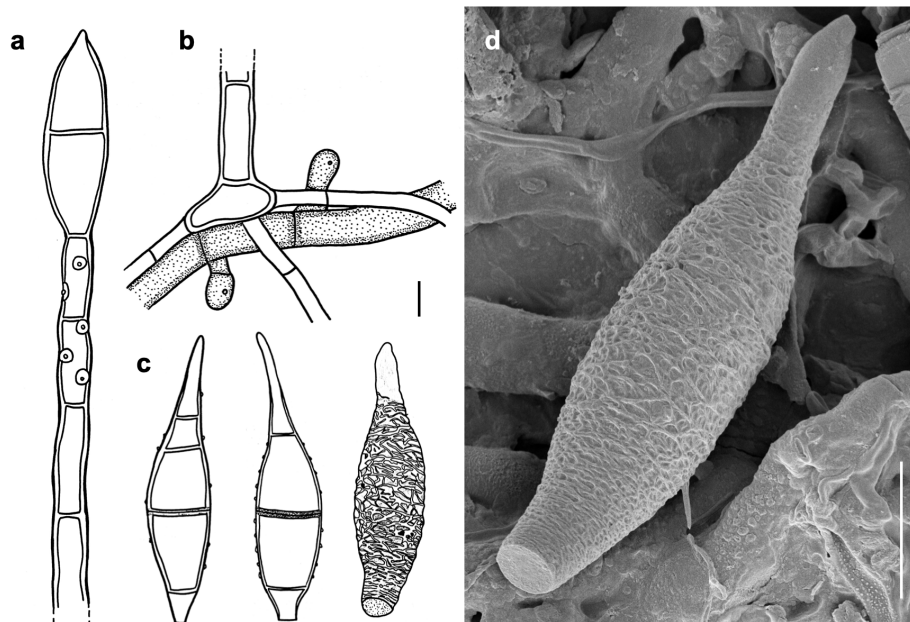
Fig. 17 *Spiropes leonensis* (IMI 46589b). a Conidiophore with scars and a young conidium, shown in optical section; **b** Part of a conidiophore growing on a hypha of *Meliola* sp., shown in optical section; **c** Conidia shown in optical section (first two drawings, from left to right), and as seen by SEM; **d** Conidium as seen by SEM. Scale bars: 8.5 µm (**a–c**); 7 µm (**d**).

Spiropes melanoplaca (Berk. & M.A. Curtis) M.B. Ellis, *Mycol. Pap.* 114: 28, 1968. (Fig. 18)

= *Arthrobotryum melanoplaca* Berk. & M.A. Curtis, *J. Linn. Soc. Bot.* 10(46): 360, 1868.
   ≡ *Podosporium melanoplaca* (Berk. & M.A. Curtis) Cif., *Sydowia* 9(1-6): 310, 1955.
= *Podosporium dialii* Bat. [as 'dialiumii'], *Atas Inst. Micol.* 1: 266, 1960. **New synonym proposed in this study.**

≡ *Spiropes dialii* (Bat.) M.B. Ellis, *Mycol. Pap.* 114: 27, 1968. **New synonym proposed in this study.**
= *Arthrobotryum scoparium* Henn., *Hedwigia* 43(6): 397, 1904. **New synonym proposed in this study.**

Colonies effuse, dark brown to black, hairy, with tightly packed hyphae that form large, erect, dark synnemata clearly visible under the stereomicroscope. **Hyphae** superficial, branched, septate, 1.5–6 μm wide, pale olivaceous, smooth. **Conidiophores** tightly packed to form dark brown to blackish synnemata up to 1.5 mm high, spreading out at the apex, 20–80 μm thick, splaying out at the apex. Individual hyphae straight or flexuous, cylindrical, 2–6 μm thick along most of their length, 5-8 μm thick near the apex, with numerous small scars that may overlap like scales. As evident by SEM, the scales are produced by the peeling of the outer wall layers where the scars are located. **Conidia** straight or curved, fusiform to obclavate, 3-septate, (30–)40–52(–68) x (7–)9–11(–14) μm, with the two middle cells usually golden brown or brown, warty, and the cells at each end paler. As seen by SEM, the ornamentation of the spores is distinctly reticulated, with thin to thick networks and no ridges.

Specimens examined – On *Meliola mangiferae* on living leaves of *Mangifera indica* (Anacardiaceae), Panama, Chiriquí Province, Los Algarrobos, 8°31'05"N; 82°25'25"W, 168 m a.s.l., 20 January 2020, M. A. Bermúdez-Cova, MB 81; same fungal and plant host, Panama, Chiriquí Province, Universidad Autónoma de Chiriquí (UNACHI), 8°25'57"N; 82°27'02"W, 37 m a.s.l., 23 January 2020, M. A. Bermúdez-Cova, MB 85; same fungal and plant host, Panama, Chiriquí Province, Los Algarrobos, Majagua river trail, 8°28'56"N; 82°24'47"W, 101 m a.s.l., 23 January 2020, M. A. Bermúdez-Cova, MB89; same fungal and plant host, Panama, Chiriquí Province, Meseta de Chorcha, 8°24'19"N; 82°13'26"W, 94 m a.s.l., 16 February 2020, M. A. Bermúdez-Cova, A. Sanjur, MB 100; same fungal and plant host, Panama, Chiriquí Province, Boquerón District, Hidroeléctrica Chuspa, 8°33'37"N; 82°36'22"W, 331 m a.s.l., 6 March 2020, M. A. Bermúdez-Cova, A. Sanjur, S. Samaniego, MB 119; On *Meliola* sp. on living leaves of *Angylocalyx oligophyllus* (Fabaceae), Benin, Attogon, Niaouli, Niaouli forest, 6°44'42"N; 2°7' 50"E, 69m a.s.l., 28 February 2022, M.A. Bermúdez-Cova, A. Tabé, I. Agonglo, O.P. Agbani, M. Piepenbring, N.S. Yorou, MB 173; on *Meliola mangiferae* on living leaves of *Mangifera indica*, Benin, Attogon, Niaouli, Niaouli forest, 6°44'44"N; 2°7'49"E, 65m a.s.l., 28 February 2022, M.A. Bermúdez-Cova, A. Tabé, I. Agonglo, O.P. Agbani, M. Piepenbring, N.S. Yorou, MB 180.

Additional specimens examined – On *Meliola mangiferae* on *Mangifera indica*, Brunei, 1974, W.T.H. Peregrine (IMI189570a); on *Meliola* sp. on *Psychotria* sp. (Rubiaceae), Cuba, 1879, C. Wright (IMI 105348 and IMI 105349, syntypes of *Arthrobotryum melanoplaca*).

Illustrations – This species was illustrated by Ellis (1968).

Known hosts and distribution – On colonies of Meliolales, especially *Meliola* spp., on living leaves of various plants in Brazil, Cuba, China, Dominican Republic, Ghana, Guadalcanal, India, Malaysia, Peru, Philippines, Sierra Leone, Tanzania, Trinidad and Uganda (Ellis 1968; Zhao et al. 1996; Dubey and Moonnambeth 2013). *Spiropes melanoplaca* is reported here for the first time for Benin and Panama.

Notes – According to Ellis (1968), the main difference between *Spiropes melanoplaca* and *S. dialii* is the range of spore width, with *S. melanoplaca* having wider spores (9-14 μm wide) than *S. dialii* (7-9 μm wide). However, after revision of several specimens and herbarium material from both species, we noticed that the aspect of the colonies, morphological features (both as seen in LM and by SEM) are similar between the species, and both species present conidia with a similar size range (Fig. 15). Therefore, we propose *S. dialii* as a synonym of *S. melanoplaca.*
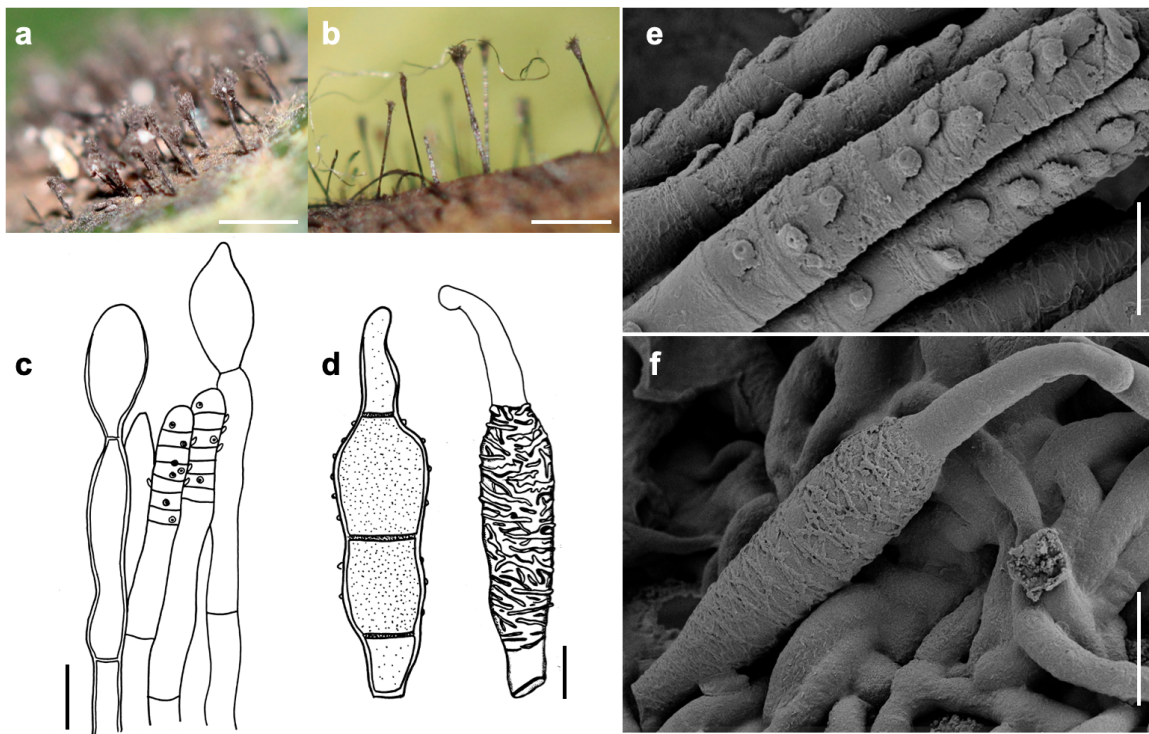
Fig. 18 *Spiropes melanoplaca* **(MB81, MB119, IMI189570a). a,b** Synnemata growing on hyphae of *Meliola mangiferae* on living leaves of *Mangifera indica*; **c** Conidiophores with scars and young conidia shown in optical section. The thickness of the wall is only shown in the first conidiophore, from left to right; **d** Conidia, shown in optical section (left-hand drawing) and as seen by SEM (right-hand drawing); **e**–**f** As seen by SEM. **e** Parts of conidiophores with scars; **f** Conidium. Scale bars: 1.5 mm (**a**); b); 0.9 mm (**c**); 8 μ (m**d**); 7 μm (**e**); 8 μm (**f**).

Spiropes palmetto (W.R. Gerard) M.B. Ellis, *Mycol. Pap.* 114: 16, 1968. (Fig. 19)

≡ *Helminthosporium palmetto* W.R. Gerard, *Grevillea* 17(83): 68, 1889.
≡ *Pleurophragmium palmetto* (W.R. Gerard) S. Hughes, *Can. J. Bot.* 36: 778, 1958.

Colonies effuse, dark brown to black, hairy. **Hyphae** superficial, branched, anastomosing, septate, 1–4 μm wide, pale olivaceous brown, smooth. **Conidiophores** arising singly or in groups, as terminal and lateral branches on the hyphae, erect, straight or flexuous, septate, up to 400 μm long, 6–10 μm thick, dark brown, paler towards the apex, smooth, with scattered conidial scars. **Conidia** solitary, obclavate to fusiform, rostrate, with 2 septa delimiting a barrel-shaped central cell, and often with an additional dark central pseudoseptum, (27–)30–46 x (7–)9–12(–15) μm, 3–5 μm wide at the truncate base, brown, middle cells pale brown, smooth as seen by LM and SEM.

Specimens examined – On *Meliola* sp. on leaves of *Elaeis guineensis* (Arecaceae), Ghana, Apremodo, 1949, S.J. Hughes 534 (IMI 38617); on *Meliola* sp. on leaves of *Sabal palmetto* (Arecaceae), U.S.A, Louisiana (IMI 10032, type of *Helminthosporium palmetto*).

Illustrations – This species was illustrated by Ellis (1968).

Known hosts and distribution – On colonies of *Irenopsis* spp. and *Meliola* spp. on living leaves of various plants in Ghana, Malaysia, New Zealand, Puerto Rico, Sierra Leone and the U.S.A. (Ellis 1968).

Notes – *Spiropes palmetto* can be easily recognized by the presence of conidia with two septa that delimit a barrel-shaped central cell and with a dark central pseudoseptum (Ellis 1968).
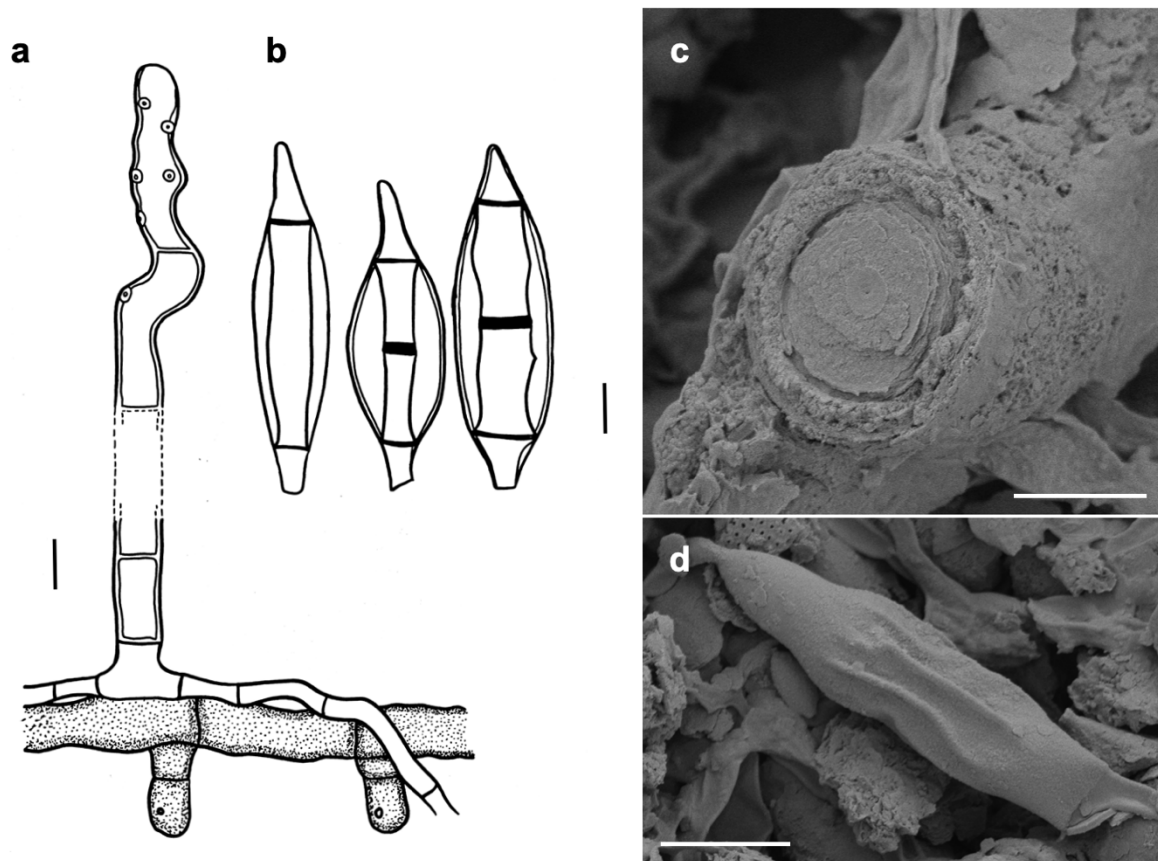
Fig. 19 *Spiropes palmetto* (**IMI 10032**). **a** Conidiophore growing on a hypha of *Meliola* sp., shown in optical section; **b** Conidia shown in optical section. The thickness of the walls is only shown in the two last drawings; **c–d** As seen by SEM. **c** Part of a conidiophore with a scar; **d** Conidium. Scale bars: 7 μm (**a**); 5 μm (**b**); 6 μm (**c**); 7 μm (**d**).

Spiropes penicillium (Speg.) M.B. Ellis, Mycol. Pap. 114: 23, 1968. (Fig. 20)

≡ *Podosporium penicillium* Speg., *Boln. Acad. nac. Cienc. Córdoba* 11: 618, 1889.
≡ *Arthrobotryum penicillium* (Speg.) F. Stevens, *Bot. Gaz.* 65: 238, 1918.
= *Arthrobotryum strychni* Henn., *Hedwigia* 43: 397, 1904.
≡ *Podosporium strychni* (Henn.) Cif., *Sydowia* 9: 311, 1955.
= *Arthrobotryum glabroides* F. Stevens, *Bot. Gaz.* 65: 237, 1918.
≡ *Podosporium glabroides* (F. Stevens) Cif., *Sydowia* 9: 309, 1955.

Colonies effuse, yellowish to dark olivaceous brown, velvety, with tightly packed hyphae that form large, erect, dark synnemata clearly visible under the stereomicroscope. A bright yellow pigment diffuses out when colonies are mounted in lactic acid or lacto-phenol. **Hyphae** superficial, branched, septate, 1–2 μm wide, yellowish, pale olive, smooth. **Conidiophores** tightly packed to form dark brown to blackish synnemata up to 650 μm long, 10–40 μm thick, often splaying out to a width of 100 μm at the apex. Individual hyphae straight or flexuous,

cylindrical, 1–2 µm thick near the base, 2–3.5 µm thick near the apex, pale olivaceous brown, smooth, with numerous small conidial scars. **Conidia** solitary, fusiform or occasionally almost cylindrical, mostly 3(–5)–septate, 16–23(–37) x (3–)3.5–5(–7) µm, tapering to about 1 µm at the apex and base, middle cells pale brown, the cells at each end paler, surface wrinkled or verruculose. As seen by SEM, the ornamentation of the spores is distinctly reticulated, with thin to thick networks that can form ridges-like structures.

Specimen examined – On *Meliola calva* on leaves of Lauraceae, Brasil, S. Paulo, Apiahy, 1881, J. Puiggari 1483 (IMI 131184, type of *Podosporium penicillium*); on *Meliola* sp. on leaves of *Oxyanthus* sp. (Rybiaceae), Sierra Leone, 1951, D.S. Rennis (IMI 51664).

Illustrations – This species was illustrated by Ellis (1968).

Known hosts and distribution – On colonies of *Asteridiella* spp. and *Meliola* spp. on living leaves of various plants in Brazil, Congo, Costa Rica, Ghana, Ivory Coast, Nigeria, Sierra Leone and Uganda (Ellis 1968).

Notes – *Spiropes penicillium* is easily distinguishable from other known synnematous species of the genus *Spiropes* by the presence of fusiform to cylindrical conidia without rostra. In addition, a bright yellow pigment diffuses out of the cells when colonies are mounted in lactic acid or lacto-phenol (Ellis 1968).
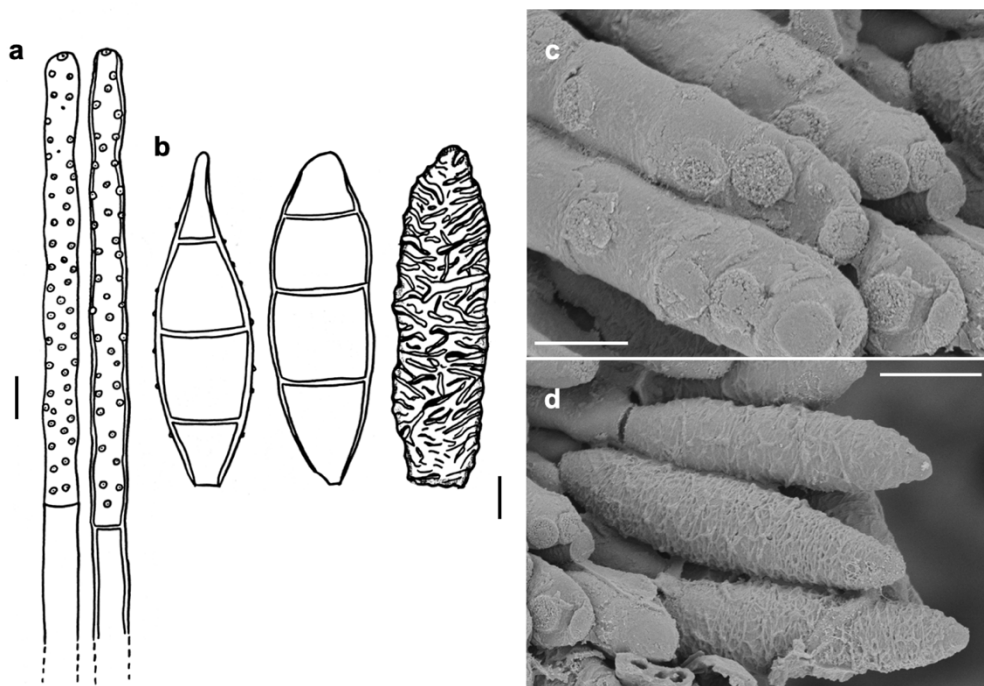
Fig. 20 *Spiropes penicillium* **(IMI 51664). a** Conidiophores with scars (the thickness of the wall is shown on the right-handed drawing); **b** Conidia shown in optical section (first two left-hand drawings) and as seen by SEM; **c–d** As seen by SEM. **c** Tips of conidiophores with scars; **d** Conidia. Scale bars: 5 μm (**a**); 2.5 μm (**b**); 3 μm (**c**); 5 μm (**d**).

Key to species of *Atractilina* and *Spiropes* hyperparasitic on Meliolales

1 Conidiophores synnematous 2
1* Conidiophores single or in groups 7

2 Synnemata straw-coloured to pale olivaceous; conidiophores with denticulate conidiogenous loci; pale multiseptate conidia .... **A. parasitica**
2* Synnemata dark brown to black; conidiophores with cicatrized conidiogenous loci; conidia pigmented and multiseptate 3

3 Synnemata up to 400 μm long; conidia mostly crescent shape .... **S. croissantiformis**
3* Synnemata longer, from 700 μm to 1.5 mm long; conidia fusiform to obclavate, occasionally cylindrical 4

4 Conidia fusiform to almost cylindrical; a yellow pigment diffuses out when colonies are mounted in lactic acid or lacto-phenol .... **S. penicillium**
4* Conidia fusiform to obclavate; no yellow pigment 5

5 Conidia always 4–6 septate .... **S. japonicus**
5* Conidia always 3–septate 6

6 Conidia 17–25 x 5–6.5 μm .... **S. clavatus**
6* Conidia 40–52 x 9–11 μm .... **S. melanoplaca**

7 Conidia with 3–6 pseudosepta 8
7* Conidia 1–3–septate 10

8 Conidiophores in larger groups; conidia with 3–6 (usually 4 or 5) pseudosepta .... **S. capensis**
8* Conidiophores single or in small groups; conidia with 3–5 pseudosepta 9

9 Conidiophores with zigzag shape; conidia with 3–5 pseudosepta, fusiform to obclavate .... **S. guareicola**
9* Conidiophores without zigzag shape; conidia with 3–4 pseudosepta, obovate .... **S. fumosus**

10 Conidia 1–septate 11
10* Conidia 3–septate 12

11 Conidia obpyriform, verrucose .... **S. armatellae**
11* Conidia obpyriform, smooth .... **S. armatellicola**

12 Conidia oblong-ellipsoid .... **S. intricatus**
12* Conidia of various shapes, not oblong-ellipsoid 13

13 Conidia obovate to clavate; conidiophores swollen towards the apex or in areas where conidia are produced .... ***S. deightonii***

13* Conidia ovate or fusiform to obclavate; conidiophores not swollen towards the apex or in areas where conidia are produced 14

14 Conidia obclavate; central cells barrel-shaped 15
14* Conidia ovate or fusiform to obclavate; without central barrel-shaped cells 16

15 Conidia with 3 true septa .... ***S. caribensis***
15* Conidia with 2 septa and a dark central pseudoseptum .... ***S. palmetto***

16 Conidia ovate .... ***S. carpolobiae***
16* Conidia fusiform to obclavate 17

17 Conidia 3–4.5 µm wide .... ***S. effusus***
17* Conidia wider 18

18 Conidia 17–25 µm long .... ***S. angylocalycis***
18* Conidia longer 19

19 Conidia 20–35 µm long .... ***S. dorycarpus***
19* Conidia longer 20

20 Conidia 36–48 µm long .... ***S. helleri***
20* Conidia 40–54 µm long .... ***S. leonensis***

In Fig. 21 we propose a visual key to the known species of *Spiropes* hyperparasitic on Meliolales.
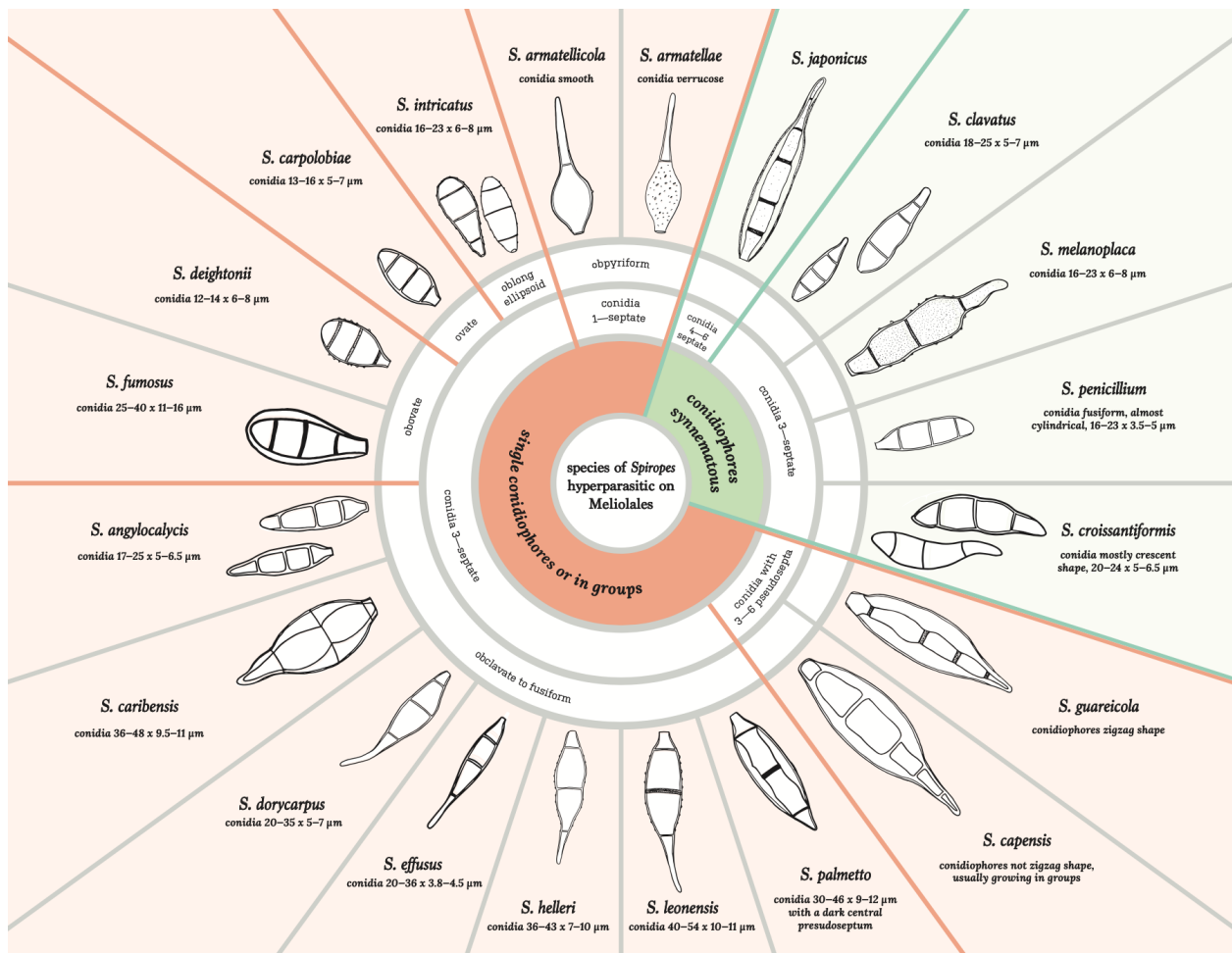
Fig. 21 Visual key to known species of *Spiropes* hyperparasitic on Meliolales.

Molecular position of species of *Atractilina* and *Spiropes*

In order to know the systematic positions of species of *Atractilina* and *Spiropes* hyperparasitic on Meliolales, new sequences of recently collected specimens were obtained.

The BLAST query revealed that the nrLSU sequences of *Atractilina parasitica* (specimens MB136 and MB178) show approximately 82 % of similarity with sequences of species of the Dothideomycetes, such as *Botryosphaeria* spp., *Helminthosporium asterinum* Cooke, *Hysterobrevium mori* (Schwein.) E. Boehm & C.L. Schoch and *Neoheleiosa lincangensis* Mortimer, among others. In the tree inferred from the analysis of LSU sequences of 45 specimens of several orders of Dothideomycetes (Fig. 22), the sequences of *A. parasitica* are located in a well- supported clade that comprises species of Pleosporales, such as *Ellismarporium parvum* R.F. Castañeda & W.B. Kendr., *Kirschsteiniothelia aethiops* (Sacc.) D. Hawksw. and *Helminthosporium asterinum*. In addition, the sequences of *A. parasitica*

cluster together in a strongly supported clade with two DNA sequences we obtained from *Malacaria meliolicola* (specimens AK4H and AK06H), a hyperparasitic perithecioid fungus that usually grows among the synnemata of *A. parasitica* on coffee leaves (see Bermúdez-Cova et al. 2023b for the updated species description of *M. meliolicola*).

As for species of *Spiropes*, the BLAST query revealed that the nrITS sequences of *Spiropes melanoplaca* (specimens MB81 and MB119) and *Spiropes japonicus* (specimen MB 120) are not closely related to each other (60 % similarity) and show between 88–90 % of similarity with species of the Leotiomycetes, such as *Lophodermium actinothyrium* Fuckel and *Hypoderma* spp., among others. Placement onto the Pezizomycotina tree version 2 in T-BAS confirmed that the newly generated ITS sequences for the two species of *Spiropes* are placed in the Leotiomycetes (Fig. 23).
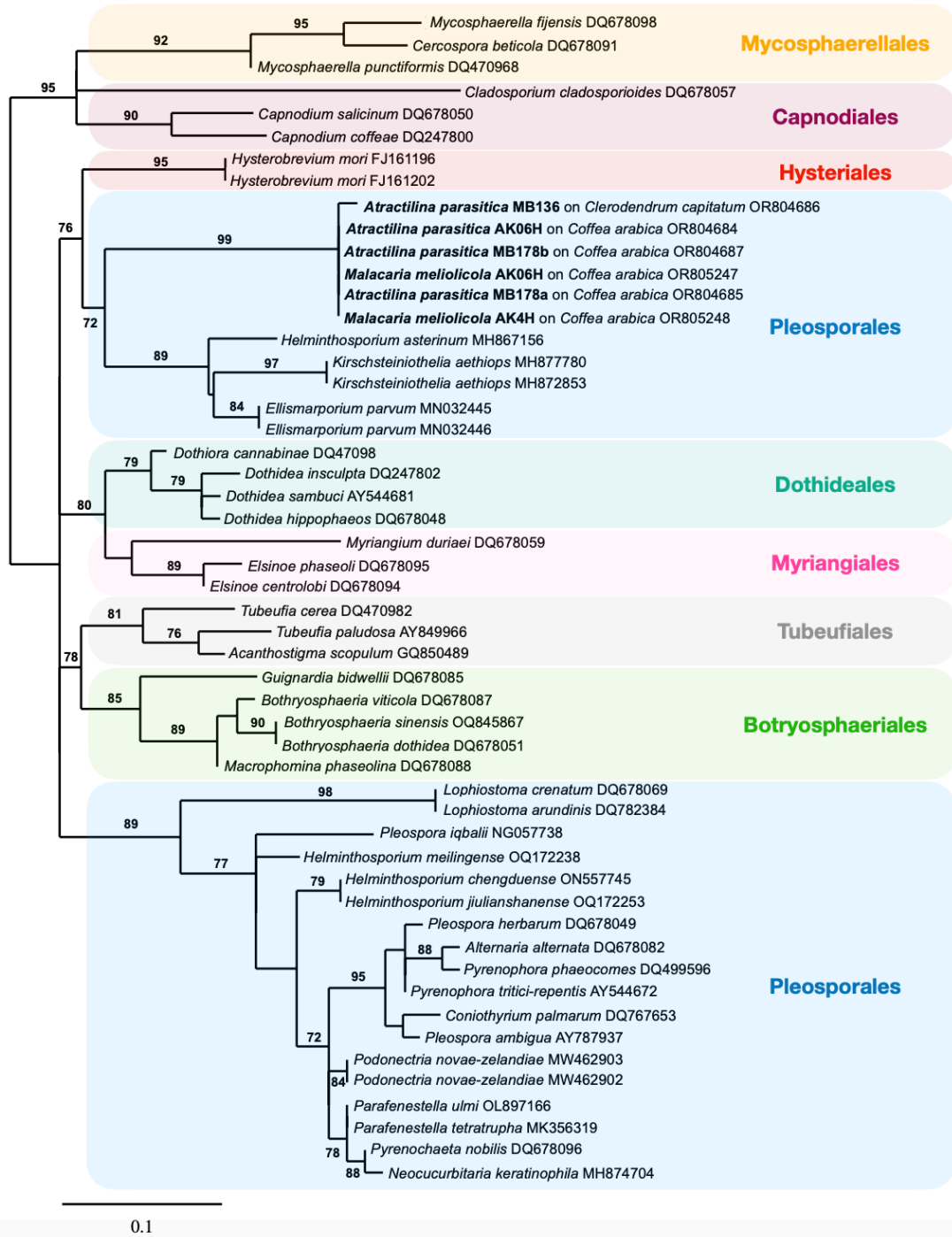
Fig. 22 Phylogenetic tree inferred from a Maximum Likelihood analysis of nuc LSU rDNA sequences of members of the Dothideomycetes, including new sequences of *Atractilina parasitica* and *Malacaria meliolicola* (written with bold letters). The tree is rooted with sequences of species of the orders Capnodiales and Mycosphaerellales. Bootstrap values are indicated above the branches. Sequences downloaded from GenBank are given with accession numbers.
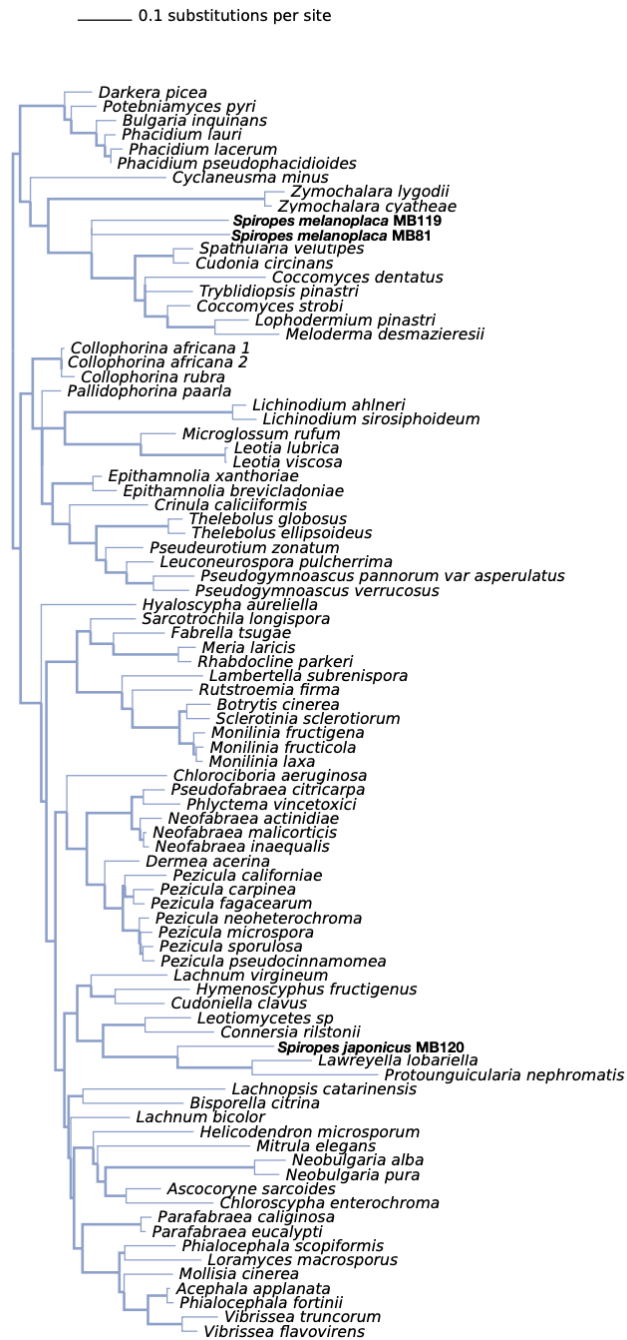
Fig. 23 Placement of *Spiropes japonicus* and *S. melanoplaca* onto Pezizomycotina reference tree version 2 in T-Bas. Only the Leotiomycetes clade is shown. The tree is the result of RAxML analysis of nuc ITS rDNA with 500 bootstraps replicates. For each node, the maximum likelihood bootstrap (≥ 70 %) is presented as thick branches. Names of *Spiropes* species with newly generated sequence data are written in bold.

Discussion

Atractilina and *Spiropes*, two genera with heterogeneous species

Morphology-based identification of a species can be very difficult, especially among asexual or non-sporulating fungi (Jeewon et al. 2002; Promputtha et al. 2005, 2007). However, it continues to be an essential tool, especially for understudied groups of fungi and when DNA sequences are not available or scarce (Raja et al. 2017). The morphological analyses and the literature review of specimens of *Atractilina* and *Spiropes* revealed that both genera include highly heterogeneous species that are not necessarily congeneric with the type species of each genus.

The type species of *Atractilina*, *Atractilina callicarpae* Dearn. & Barthol. (= *Atractilina parasitica* (G. Winter) Deighton & Piroz.), has consistently true synnematous conidiophores, denticulate conidiogenous loci, pale pluriseptate (phragmoseptate) conidia and a hyperparasitic lifestyle (Deighton and Pirozynski 1972; Mel'nik and Braun 2013). Based on these characteristics, only three species of the genus are congeneric with *A. parasitica*, namely *A. alinae* Melnik & U. Braun, *A. biseptata* R.F. Castañeda and *A. calycini* T.K. Jana, S.N. Ghosh & A.K. Das (Castañeda-Ruiz 1986; Jana et al. 2006; Mel'nik and Braun 2013). The remaining two species present non-synnematous conidiophores and are probably not congeneric. *Atractilina asterinae* (Hansf.) Deighton & Piroz. is a species hyperparasitic on Asterinales, and presents single conidiophores and distoseptate conidia (Deighton and Pirozynski 1972). *Atractilina hymenaeae* Bat. & J.L. Bezerra (introduced as *Atractina hymenaeae* by the authors) is hyperparasitic on Meliolales, but also with non-synnematous conidiophores and conidia with a variable number of septa (Batista and Bezerra 1961). Therefore, we believe that both species have been incorrectly assigned to the genus *Atractilina*.

The description of *A. parasitica* introduced by Deighton and Pirozynski (1972) is very broad. As a result, specimens with significant morphological variations are grouped into a single species concept. For example, Chen and Tzean (2007) described a parasitic fungus from Taiwan growing on decaying leaves of *Liquidambar* sp. (Altingiaceae), with conidia that resemble those of *A. parasitica*. However, conidiophores of this fungus are non-synnematous and very short (less than 15 μm long), a feature that has never been reported before for *A. parasitica*. It is necessary to re-evaluate this and other identifications, to narrow the species concept of *A. parasitica*, as well as to complement it with DNA sequence data.

The DNA molecular analyses of the nrLSU rDNA region of the specimens of *A. parasitica* from Benin revealed that this species belongs to the Dothideomycetes. The Dothideomycetes

are the largest and most diverse class of fungi, and comprise species that exhibit a broad range of lifestyles, including saprotrophs, plant pathogens, mycoparasites and hyperparasites, as well as lichenized and lichenicolous fungi (Pem et al. 2021). They typically produce flask-like structures called pseudothecia, though apothecial, hysterothecial and cleistothecioid ascomata also exist (Hessen and Jahns 1973; Valenzuela-Lopez et al. 2019). Bitunicate asci are one of the diagnostic characters for Dothideomycetes taxonomy (Von Arx and Müller 1975; Pem et al. 2021). Asexual stages are frequent among pathogenic genera in the families *Cladosporiaceae*, *Mycopsphaerellaceae*, *Pleosporaceae* and *Tubeufiaceae*, among others (Hyde et al. 2013; Wanasinghe et al. 2018; Hongsanan et al. 2020). Conidiophores in these anamorphic species are usually solitary or in groups forming synnemata (Thambugala et al. 2017). The sequences of *A. parasitica* showed 98 % of similarity with sequences of *Malacaria meliolicola* (Dothideomycetes, Ascomycota), a pseudothecioid hyperparasite that was found repeatedly among the synnemata of *A. parasitica* (Bermúdez-Cova et al. 2023b). Therefore, the systematic position of *A. parasitica* in the Dothideomycetes and the anamorph-teleomorph connection between these two species are confirmed. This connection has been proposed in the past for these fungi on leaves of *Coffea arabica* (Hansford 1941, 1946, Bermúdez-Cova et al. 2023b). Here, a DNA sequence from a specimen of *A. parasitica* on *Meliola* sp. on leaves of *Clerodendrum capitatum* clustered with the aforementioned sequences in a highly supported clade. The phylogenetic analysis of the nrLSU DNA locus showed that sequences of *A. parasitica* are located in a well-supported subclade together with other species of Pleosporales s.l., such as *Ellismarsporium parvum* (Zang et al. 2020). Many species of the Dothideomycetes, especially the asexual genera, are known to be polyphyletic (Schoch et al. 2009). To confirm the systematic hypothesis and to determine the placement of *A. parasitica* at family level, the use of multi-loci phylogenies is necessary in the future.

As for the genus *Spiropes*, the generic diagnosis given by Ellis (1968, 1971) allows to include in this genus all species with cicatrized conidiogenous cells and conspicuous, flat and numerous scars, as well as pigmented, mostly obclavate phragmoconidia with 1–9 septa or pseudosepta. Seifert and Hughes (2000) proposed an amendment of this generic concept to also include species with dictyoconidia. As a result, *S. dictyosporus* is the only known species of the genus with muriform conidia. However, this morphological diagnosis allows for species with a wide range of types of conidiophores, conidiogenesis and conidia to be included in *Spiropes* (McTaggart et al. 2007). For example, the type species of the genus, *Spiropes guareicola* (F. Stevens) Cif., has distinctly sympodial-geniculate (zigzag-shaped) conidiophores, a character

that is not present in any other known species of the genus (Ellis 1968). This species, in addition, presents distoseptate conidia, i.e., conidia with pseudosepta, a morphological feature that is present only in four species, namely *S. capensis*, *S. fumosus, S. guareicola* and *S. japonicus*. The remaining species of the genus present euseptate conidia (Ellis 1968, 1971). It is also possible to find a wide range of conidial shapes, such as obpyriform, obovate, ovate and oblong ellipsoid, to obclavate and fusiform (see the visual key to species of *Spiropes* in Fig. 21). Therefore, *Spiropes* is currently a genus with morphologically highly heterogeneous species and probably polyphyletic.

Identifying species of *Spiropes* based on morphology alone is not always easy. The most comprehensive key to species of the genus was proposed by Ellis (1968). However, this key is mainly based on the differences in the size range of the conidia of the species and, in some cases, these size differences are very subtle. Particular attention should be paid to herbarium specimens, as they may include immature or not well-preserved spores that can affect measurement results (Ordynets et al. 2021). We believe that other morphological characteristics that are not visible using standard light microscopy techniques should be considered when identifying species of *Spiropes* (e.g., Lutzoni et al. 2004). Scanning electron Microscopy (SEM), for example, allowed us to observe for the first time the surface of the conidia of species of *Spiropes*. *Spiropes diallii* and *S. melanoplaca* were considered as different species by Ellis (1968). However, both species have overlapping spore size ranges, and the morphological analysis by SEM revealed that these species also have similar conidiogenesis and ornamentation patterns on conidia. This situation is similar for *S. intricatus* and *S. pirozynskii.* Therefore, we propose both groups of species as synonyms.

As for the molecular-based identification of species of *Spiropes*, there are currently no DNA sequences available in publicly accessible databases. Species of the genus remain "incertae sedis" for many taxonomic ranks and it is difficult to assign new DNA sequences to species concepts (Bermúdez-Cova et al. 2022, 2023a). The DNA sequences generated for the first time in the context of this study suggest that species of *Spiropes* hyperparasitic on Meliolales may be polyphyletic in the Leotiomycetes. Fungi in the class Leotiomycetes are ecologically diverse and have been described as aquatic hyphomycetes, ectomycorrhizal parasites, endophytes, fungal parasites, mycorrhizal fungi, nematode-trapping fungi and plant-pathogens, among others (Wang et al. 2006a; Johnston et al. 2019). Many fungi have been suggested to belong to this class without any clear teleomorphic connection (Wang et al. 2006b). Up to date, no sexual

stages have been linked to any species of *Spiropes* (Bermúdez-Cova et al. 2022). There is one genus with species morphologically similar to species of *Spiropes*, namely *Pseudospiropes* M.B. Ellis (Helotiales, Leotiomycetes; Ellis 1971). Species of this genus differ from species of *Spiropes* by broadly enlarged, thickened, protuberant, strongly melanized conidiogenous loci and distoseptate conidia only (Castañeda-Ruiz et al. 2001; McTaggart et al. 2007). Species of *Pseudospiropes* have *Strossmayeria* Schulzer (Helotiales, Leotiomycetes) teleomorphs (Iturriaga and Korf 1984, 1990; Castañeda-Ruiz et al. 2001). Thus, there is a possibility that species of the genus *Spiropes* also belong to the Leotiomycetes. It is necessary to continue generating new DNA sequences from the different species of the genus in order to confirm this hypothesis, especially from those species that form part of mixed infections.

The need for reevaluation, resampling and epitiypification

Applications of names based on morphological characteristics without DNA data is a challenge, resulting in the description of an excessive number of species or, in contrast, in the overlooking of cryptic species that can only be detected through molecular analyses (Hibbett et al. 2007, Crous et al. 2014, Jayasiri et al. 2015). The knowledge of morphological characteristics, however, is important to understand the evolution of fungal diversity (Raja et al. 2017). Instead of describing new species as part of *Atractilina* and *Spiropes*, a re-evaluation of the natural concepts of both genera is needed. Here we propose a list of actions that are necessary to carry out such a re-evaluation:

- Restudy the type species of each genus. When the type specimens of the type species are not in good condition or there is no more fungal material available for examination, it is necessary to recollect them. Epitypes and neotypes should be designated in these cases.
- After redefining the type species, all species belonging to the two genera need to be recollected, reanalyzed morphologically and compared to the type species.
- The DNA of all existing species should be extracted, amplified and sequenced, in order to confirm or propose new concepts of genera and species. Multi loci phylogenetic analyses are necessary to validate or propose new systematic hypotheses.

Atractilina and *Spiropes* are currently two repository genera of highly heterogeneous species, and they may be split in the future, once species and genus concepts are validated respectively by morphology and molecular methods.

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