Laboulbeniomycetes, Enigmatic Fungi With a Turbulent Taxonomic History☆

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From Roland Thaxter to the Present: Synergy Among Mycologists, Entomologists, Parasitologists

Laboulbeniales were discovered in the middle of the 19th century, rather late in mycological history (Anonymous, 1849; Rouget, 1850; Robin, 1852, 1853; Mayr, 1853). After their discovery and eventually their recognition as fungi, occasional reports increased species numbers and broadened host ranges and geographical distributions; however, it was not until the fundamental work of Thaxter (1896, 1908, 1924, 1926, 1931), who made numerous collections but also acquired infected insects from correspondents, that the Laboulbeniales became better known among mycologists and entomologists. Thaxter set the stage for progress by describing a remarkable number of taxa: 103 genera and 1260 species.

Fewer than 25 species of *Pyxidiophora* in the Pyxidiophorales are known. Many have been collected rarely, often described from single collections and never encountered again. They probably are more common and diverse than known collections indicate, but their rapid development in hidden habitats and difficulty of cultivation make species of *Pyxidiophora* easily overlooked and, thus, underreported (Blackwell and Malloch, 1989a,b; Malloch and Blackwell, 1993; Jacobs *et al.*, 2005; Gams and Arnold, 2007). The group is crucial, however, because of its taxonomic position within the Laboulbeniomycetes to provide a morphological link between the thallus-forming Herpomycetales and Laboulbeniales (Haelewaters *et al.*, 2021c) and perithecial ascomycetes (**Fig. 1**).

Cooperation among entomologists, botanists, and parasitologists easily goes back as far as the mid-19th century as evidenced by the correspondence of Charles Darwin and Henry Denny, an entomologist and authority on parasites (Darwin, 1865). Likewise, Thaxter's major contributions were possible not only from his own field collections and museum visits, but also from specimens sent to him by a wide network of entomologists. These contacts provided access to many hosts from places never visited by Thaxter, such as the African continent and southeastern Asia, from which he described many taxa. After Thaxter's death, most of the Laboulbeniomycetes literature concerned regional studies and parasite–host lists in different geographical regions, including Belgium and the Netherlands (De Kesel and Rammeloo, 1992; De Kesel *et al.*, 2020; Haelewaters and De Kesel, 2020); Finland (Huldén, 1983); Germany (Scheloske, 1969); Poland (Majewski, 1994, 2003), the Iberian Peninsula (Santamaría, 1998, 2003), and Japan (Sugiyama, 1973). Many species reported by these authors continue to result from hosts provided by entomologists or from museum insect collections (e.g., Santamaría *et al.*, 2016; Haelewaters and Rossi, 2017; Kaishian and Weir, 2018; De Kesel and Haelewaters, 2019; Kaishian *et al.*, 2020; Rossi and Leonardi, 2020). Currently, projects focusing on Laboulbeniales associated with the bat fly parasites of bats, revive the tradition of collaboration among mycologists, entomologists, and even mammalogists (Walker *et al.*, 2018; Haelewaters *et al.*, 2021a).

The major contribution of Benjamin (1971) made the massive work of Thaxter more accessible; he also reviewed his own work and the research of the post-Thaxer half century. Fifty years later, Haelewaters *et al.* (2021c) reviewed the developments in Laboulbeniomycetes of yet another half century.

The Winding Road to Molecular Phylogenetics: Progress in the Study of Laboulbeniomycetes

Advancement in the study of Laboulbeniales was initially slow because of their minute size, limited morphological traits to distinguish them among themselves, inability of most taxa to grow in axenic culture, and absence of comparative traits to place them among other fungi. Microscopes provided early evidence of the existence of Laboulbeniales, previously unknown minute ectoparasites of arthropods, and led to their recognition as fungi. After their fungal character was confirmed, better communication and transportation means enabled the discovery of additional species and broadened geographical and host ranges. Transmission electron microscopy (TEM), although less successfully applied to Laboulbeniomycetes compared to other groups of fungi, brought critical proof of free cell formation involving an ascospore-delimiting membrane in ascosporogenesis, the hallmark of the Ascomycota (Hill, 1977). Even limited success at cultivation has helped to cast light on nutritional requirements of certain Laboulbeniomycetes (Whisler, 1968; Blackwell and Malloch, 1989b; Jacobs *et al.*, 2005).

☆We dedicate this chapter to Dr. Donald H. Pfister, Curator of the Farlow Reference Library and Herbarium of Cryptogamic Botany and Asa Gray Professor of Systematic Botany at Harvard University. For almost half a century, he has been committed to preserving critical research materials and promoting their use while conducting his own extensive research and mentoring students of all ages.

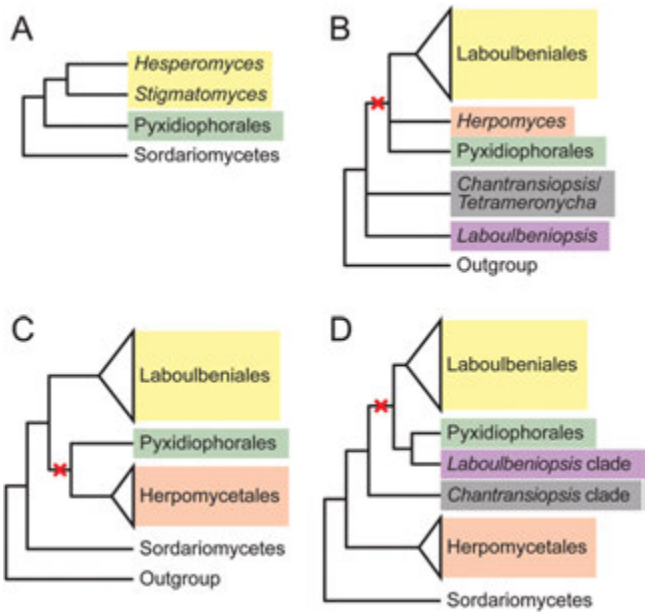

Fig. 1 Evolutionary hypotheses about the relationships between clades within the class Laboulbeniomycetes. **A.** Six-locus phylogeny based on 434 isolates, including 4 Laboulbeniomycetes isolates (modified from Schoch *et al.*, 2009). **B.** SSU rDNA phylogeny based on 65 isolates (modified from Goldmann and Weir, 2018). **C.** Three-locus rDNA phylogeny based on 61 isolates (modified from Haelewaters *et al.*, 2019d). **D.** Two-locus rDNA phylogeny based on 75 isolates (modified from Blackwell et al. 2020). Nodes without support are marked with a red "×".

Despite the discovery of free cell formation, disputes over the taxonomic position of the Laboulbeniales persisted (Cavalier-Smith, 1998; 2000), until the development of the polymerase chain reaction (PCR). Since the mid-1990s, DNA characters and ever-increasing taxon sampling have begun to unravel evolutionary relationships of Laboulbeniomycetes, to gain a better picture of their diversity, and to delineate species with innumerable discrete characters compared to those provided by morphology (e.g., Blackwell, 1994; Weir and Blackwell, 2001b; Goldmann and Weir, 2012; 2018; Haelewaters *et al.*, 2018, 2019d, 2021c; Blackwell *et al.*, 2020). Based on sequences of the small subunit (SSU) of the ribosomal RNA gene (rDNA), Weir and Blackwell (2001b) rejected a Cavalier-Smith (1998, 2000) hypothesis and showed Laboulbeniales + Pyxidiophorales to be a strongly supported single clade, class Laboulbeniomycetes, within the Ascomycota. However, no assessment of the relationship of the class among other ascomycetes was possible due to lack of support primarily due to absence of adequate sampling of ascomycete taxa. Schoch *et al.* (2009) presented the phylogenetic reconstruction of an Ascomycota-wide six-locus dataset and found strong support for the sister relationship of the classes Laboulbeniomycetes and Sordariomycetes, suggestive of a single origin of perithecial fungi.

Based on SSU rDNA, Goldmann and Weir (2018) published the taxonomically broadest molecular phylogeny of the Laboulbeniomycetes to date. Several lineages were supported within the class and the problematic filamentous conidial insect ectoparasites, *Chantransiopsis* and *Tetrameronycha*, were included in the class. In addition, a sequence of *Herpomyces* from Haelewaters *et al.* (2015b) fell outside of the Laboulbeniales in an unresolved position based on Bayesian inference or in an unsupported clade with *Laboulbeniopsis* using maximum likelihood criteria. Goldmann and Weir (2018) considered this analysis to be supportive of the placement of *Herpomyces* in the suborder Herpomycetinae (*fide* Tavares, 1985). A three-locus phylogenetic reconstruction led Haelewaters *et al.* (2019d) to elevate Herpomycetinae to order level. This move resulted in recognition of three major lineages in the class Laboulbeniomycetes: Herpomycetales, Laboulbeniales, and Pyxidiophorales.

Members of Laboulbeniales and Herpomycetales, arthropod biotrophic ectoparasites, are characterized by the formation of a non-hyphal, three-dimensional thallus of up to a few thousand cells. In contrast, species of arthropod-dispersed Pyxidiophorales are dependent on other fungi for enhanced growth or as hosts for mycoparasites; they develop hyphae and produce perithecia. It is interesting to note that because Herpomycetales and Laboulbeniales do not form a monophyletic lineage, the thallus may have originated independently in these two orders (**Fig. 1**; Blackwell *et al.*, 2020; Haelewaters *et al.*, 2021c). There are, however, some species of *Pyxidiophora* known to produce 3-dimensional cell divisions resulting in limited parenchymatous areas in parts of the mycelium and in older ascospore-derived conidial states attached to mites in moist chambers (Blackwell and Malloch, 1989b). The three formally described orders and two informal clades supported by DNA analysis are discussed below.

Thallus-Forming Ectoparasites

Laboulbeniales

The order Laboulbeniales with about 2325 described species in 145 genera (Kirk, 2019; Haelewaters *et al.*, 2020a) forms the most diverse fungal lineage associated with Arthropoda, predominantly insects (subphylum Hexapoda). These fungi occur selectively on the following insects: ants (order Hymenoptera: family Formicidae), beetles (order Coleoptera), cockroaches and termites (order Blattodea), crickets and allies (order Orthoptera), earwigs (order Dermaptera), flies (order Diptera), lice (order Psocodea), thrips (order Thysanoptera), and true bugs (order Hemiptera). Numerous other arthropods are also known to host Laboulbeniales, including millipedes (subphylum Myriapoda: class Diplopoda), harvestmen (subphylum Chelicerata: order Opiliones), and mites (subphylum Chelicerata: subclass Acari) (Weir and Hammond, 1997; Santamaría *et al.*, 2017; Haelewaters *et al.*, 2019d). Although beetles are parasitized by approximately 80% of described species (Weir and Hammond, 1997), they host a relatively small number of genera of Laboulbeniales (Haelewaters *et al.*, 2019b). Fewer species have been described from other groups, such as the Hemiptera (true bugs), with only 96 described species occurring across the suborder Heteroptera (Benjamin, 1967; Santamaría, 2008; Lee and Na, 2009; Kaishian and Weir, 2018; Kaishian *et al.*, 2020).

First sightings

The study of Laboulbeniales started with observations of *thalli* (multicellular units with determinate cell number) of *Laboulbenia* on carabid beetles in the 1840s and early 1850s (Anonymous, 1849; Rouget, 1850; Mayr, 1853). Some authors thought that the structures they observed were insect parts (Mayr, 1853) whereas others recognized them as living organisms. In those days, researchers referred to Laboulbeniales as "parasitic plants" (Anonymous, 1849) or even acanthocephalan worms (Kolenati, 1857). Robin (1852) was the first to recognize them as fungi, and de Bary (1884) listed the family Laboulbeniaceae as ascomycetes with doubt. The first to use the name "Laboulbeniaceae" was Peyritsch (1873). Five genera were recognized at that time – *Chitonomyces*, *Heimatomyces*, *Helmintophana* [= *Arthrorhynchus*], *Laboulbenia*, and *Stigmatomyces* – and twelve species had been described, eight of which were in the genus *Laboulbenia* (Benjamin, 1971). By the time Thaxter published his first *Contribution towards a monograph of the Laboulbeniaceae* (Thaxter, 1896), there were 28 genera and 152 species, most of which had been published in a series of preliminary papers (Thaxter, 1891, 1892, 1893, 1894, 1895).

Current versus estimated species diversity

While only 2325 species have been described thus far, many more species are expected to be discovered in years to come. A formal estimation of the species richness of Laboulbeniales was based on the results of a beetle survey in Sulawesi, Indonesia, in which 80,000 beetles were screened for the presence of Laboulbeniales to yield an overall prevalence of 0.6% (Weir and Hammond, 1997). The principal study area was a 500-ha patch of lowland rainforest. Of a total of 4026 beetle species, 127 were hosts of Laboulbeniales fungi (3.15%). Based on their data and comparisons with other studies (Huldén, 1983; Lee, 1986; Santamaría *et al.*, 1991; Majewski, 1994; Weir, 1996), the authors asserted that greater Laboulbeniales diversity is associated with moist tropical areas and beetle hosts. Provided that the number of beetle species is estimated to be 2 million and that the prevalence of Laboulbeniales on beetles is about 3%, Weir and Hammond (1997) inferred that the number of Laboulbeniales species on beetles would be 60,000. If the ratio of host species to parasite species is 2:1 (meaning that some species of Laboulbeniales are associated with multiple host species), this results in an estimated 30,000 Laboulbeniales species on beetles. Given that at least 75% of infections occur on beetles, one could assume that the total estimate of species in the order is 40,000 [15,000 – 75,000].

There have been few other detailed studies of Laboulbeniales at a given site. Huldén (1983) surveyed the Laboulbeniales of Finland and adjacent regions of what was formerly the U.S.S.R. based on the study of museum collections. About 160,000 insects representing 1100 species were screened for the presence of Laboulbeniales. A total of 166 insect species (beetles and flies) were found to be host to 88 species of Laboulbeniales, 24 of which were newly described. The overall prevalence for this study was approximately 1%, which is thought to be low. For example, in Central Europe, the rate of infection ranges from 10% to 35% (Huldén, 1983). This striking difference may be attributed to changing climatic conditions leading to higher winter mortality of potential hosts, in addition to smaller, more isolated host populations that overlap less frequently. Wide-scale studies of neotropical Laboubeniales diversity are lacking compared to studies from temperate regions, and no comprehensive site-based study of the group has been published from the Neotropics, although one long-term inventory project has been initiated in Cusuco National Park, Honduras (Haelewaters *et al.*, 2021b). Such studies will result in a substantial addition of new biodiversity data.

The advent of molecular studies has revolutionized our understanding of organismal relationships. These techniques are of particular value when investigating cryptic species and could help sharpen estimates of species numbers within the group. However, the isolation of DNA from thalli of Laboulbeniales was an early problem to overcome, due to their inability to grow in artificial culture, minute size, and melanized tissue (Weir and Blackwell, 2001a; Haelewaters *et al.*, 2015b; Sundberg *et al.*, 2018a). It does not come as a surprise that only 12 of 174 species of Laboulbeniomycetes described between 2010 and 2020 were accompanied by sequences (**Fig. 2**), mostly of nuclear rDNA regions, but also mitochondrial small subunit rDNA and translation elongation factor 1α gene. As integrative taxonomy practices become commonplace in species delimitation of many groups of fungi, Laboulbeniales researchers have struggled to keep pace. However, molecular phylogenetic data have successfully clarified relationships at lower taxonomic ranks (e.g., Weir and Hughes, 2002; Goldmann and Weir, 2012; Goldmann *et al.*, 2013; Sundberg *et al.*, 2018b; Haelewaters *et al.*, 2019a; Liu *et al.*, 2020) and revealed the existence of cryptic diversity in the

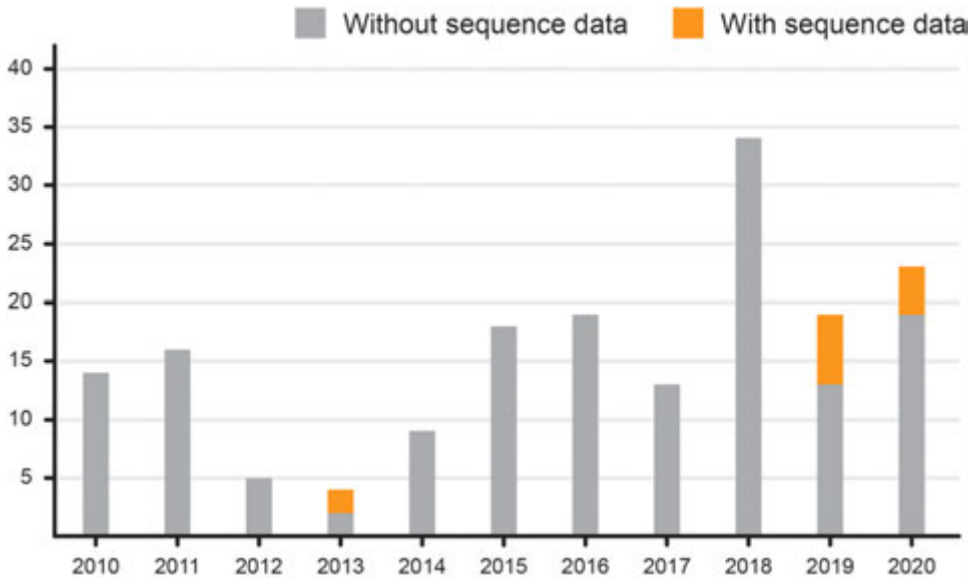

Fig. 2 Number of described Laboulbeniomycetes species – including *formae* – since 2010, based on morphology alone (without sequence data) versus using an integrative taxonomy approach (with sequence data).

Laboulbeniales, with *Arthrorhynchus eucampsipodae*, *Hesperomyces virescens*, and *Laboulbenia flagellata* having been recognized as species complexes (Haelewaters *et al.*, 2018, 2019b, 2020a; De Wegghelaire, 2019). These findings make the 40,000-figure from Weir and Hammond (1997) a conservative estimate for species richness in the order.

Still, generating sequences for Laboulbeniales remains a challenge, especially for material from dried museum collections or specimens collected >10 years ago. This dilemma necessitates consideration of the value of alpha-taxonomy. While researchers of certain groups, such as the fleshy mushrooms, may challenge the validity of species descriptions made in the absence of molecular characters, morphological descriptions have been critical in building foundational knowledge of Laboulbeniales. And while integrated taxonomy – the use of combined morphological, ecological, geographical, and molecular data – is an ideal to which researchers strive. Hibbett *et al.* (2016) pointed out that many researchers lack access to sufficient funding or equipment to generate molecular data. These researchers are often based in tropical areas where most of the world's undescribed species reside. Because millions of fungal taxa remain undiscovered, it seems prudent for taxonomists to continue working using available resources with the understanding that future molecular phylogenetic work may confirm or shift species limits. Concurrently, collaboration between fungal molecular systematists and classically trained taxonomists should be the end goal.

Museum collections, citizen science projects, and social media

There is a large discrepancy between the number of described species of fungi (138,000, Kirk, 2019) and the number of estimated species (2.2–6 million, e.g., Hawksworth and Lücking, 2017). A common inquiry asks, *where are the millions of missing fungi?* Wijayawardene *et al.* (2020) put forward five sources of undescribed fungi: (1) habitats that are naturally diverse but poorly studied, (2) cryptic taxa, (3) fungal collections that might contain cryptic or new species hidden under current names, (4) molecular novelties, and (5) natural history collections including plant herbaria and entomological collections. Indeed, insect collections are a treasure trove for Laboulbeniales researchers. Thalli of Laboulbeniales persist indefinitely on the host body whether the host is preserved in ethanol or dried and pinned. Because museum collections often contain a wide array of taxa from a broad range of localities, researchers utilizing collections can pursue a research scope focusing on specific geographical areas and/or taxa. Making use of museum collections for research as opposed to conducting fieldwork has logistical advantages, saving time and expenses, and circumventing the need to kill hundreds to thousands of insect specimens to find only a few ones hosting Laboulbeniales. Another huge advantage is that the host is often already identified by an expert.

Collections have been used to investigate an array of questions about biodiversity, taxonomy, biogeography, and host usage patterns of Laboulbeniales. Blackwell (1980a,b) screened 2517 nycteribiid bat flies at the Natural History Museum (London) and found thalli of *Arthrorhynchus* on 56 specimens. She used this material along with Thaxter's slide mounts from the Farlow Herbarium to study fungal development and morphology, host associations, and within-species phenotypic plasticity. Haelewaters *et al.* (2015c, 2019b) reported nine new country records of Laboulbeniales (Canada, USA, Croatia, Slovenia, Ukraine, DR Congo) based on dried collections of Carabidae, Coccinellidae, and Staphylinidae from the Harvard Museum of Comparative Zoology, the American Museum of Natural History (New York), Tupper Center of the Smithsonian Tropical Research Institute (Ancon, Panama), and the Collection d'insectes du Québec (Canada). Kaishian and Weir (2018) and Kaishian *et al.* (2020) described eight new species of the genera *Laboulbenia* and *Prolixandromyces* based on material from the collection of Dr. John T. Polhemus, Department of Entomology, Smithsonian National Museum of Natural History (Washington, D.C.). Santamaría *et al.* (2016)

utilized the millipede collection preserved at the Natural History Museum of Denmark (Copenhagen) to describe nine new species of *Rickia*. Haelewaters *et al.* (2017) proposed that a lag time occurred between establishment of the invasive alien ladybird *Harmonia axyridis* in nature and acquisition of *Hesperomyces virescens* by this host. The authors based their findings on the study of 7404 ladybirds collected in 1991–2015, of which 521 were from eleven museum collections in North America and Asia.

Contributions from citizen scientists have been of great importance to biodiversity research since the 19th century. Citizen science projects have gained more traction in recent years and examples of large-scale projects dependent on input from non-professionals, are commonplace (e.g., Douglas, 2016). Haelewaters *et al.* (2019c) downloaded and curated North American occurrences of *Hesperomyces virescens* associated with the invasive alien species *Harmonia axyridis* from citizen science platforms Bugguide and iNaturalist. All records were used to build a map at Beetlehangers.org (see "Relevant Websites section"), the primary aim of which is to track the distributional range of the ladybird–parasite association through time. As another example, Báthori *et al.* (2017) reported a new country record of *Rickia wasmannii* from Greece, which was found and identified using the digital image collection at AntWeb (see "Relevant Websites section"). Finally, Santamaría *et al.* (2020b) described a new species of *Troglomyces*, initially discovered in a photograph of a millipede shared on Twitter (see "Relevant Websites section") and then found on millipedes at the Natural History Museum of Denmark and the Muséum National d'Histoire Naturelle in Paris. These recent studies also speak to the timelessness of natural history collections and their continued value in modern research.

Classification of the order

Roland Thaxter not only contributed invaluable taxonomic additions, he was also the first – and for a long time the only one – to propose a classification system for the order. At the time of the first volume of his *Contribution towards a monograph of the Laboulbeniaceae* (Thaxter, 1896), what we currently refer to as the Laboulbeniales was designated a family, Laboulbeniaceae. Thaxter (1896) split up the Laboulbeniaceae into two "groups", the Exogenae and Endogenae. Development of spermatia, gametes produced on the appendages, was the sole criterion for grouping of taxa. The Exogenae included genera with species that form spermatia exogeneously. They are borne on intercalary cells or terminally on short branchlets. Only the genera *Ceratomyces* and *Zodiomyces* were part of this Exogenae group. The Endogenae comprised taxa in which spermatia are formed within antheridia. This group included two "orders": Laboulbenieae (with simple antheridia, 15 genera) and Peyritschielleae (with compound antheridia, 11 genera).

Thaxter (1908) accepted the ordinal name Laboulbeniales, which included the recently described genus *Herpomyces* (Thaxter, 1902), and replaced the terms Exogenae and Endogenae by subordinal names Laboulbeniinae and Ceratomycetinae. The two subdivisions of the original "group" Endogenae were replaced by families Laboulbeniaceae and Peyritschiellaceae. Thaxter did not recognize a family within the Ceratomycetinae. The name Ceratomycetaceae, now widely accepted, was introduced by Maire (1916) as a *nomen nudum*, later validly published by Colla (1934). This scheme of organizing taxa was widely accepted until Tavares (1967, 1985) introduced new characters for classification of the Laboulbeniales: perithecial development and perithecial wall structure. Later, Goldmann and Weir (2018) found that the number of perithecial wall cells is phylogenetically informative across the order Laboulbeniales; the authors described a progressive reduction of number of perithecial wall cells in the four vertical rows. Thaxter's (1908) two suborders, two families and twenty tribes were reorganized to two suborders, four families, six subfamilies, 13 tribes and 28 subtribes in Tavares' (1985) classification system.

Tavares (1985) recognized three families in the suborder Laboulbeniinae: Ceratomycetaceae, Euceratomycetaceae, and Laboulbeniaceae (Majewski, 1994; Santamaría, 2003). Ceratomycetaceae comprises twelve genera: *Autoicomyces*, *Ceratomyces*, *Drepanomyces*, *Eusynaptomyces*, *Helodiomyces*, *Phurmomyces*, *Plectomyces*, *Rhynchophoromyces*, *Synaptomyces*, *Tettigomyces*, *Thaumasiomyces*, and *Thripomyces*. Synapomorphic characters are (1) the primary receptacle consisting of a single series of superposed cells and (2) cells VI and VII being successive, intercalary cells of the primary receptacle (Tavares, 1985). In the Euceratomycetaceae, cells VI and VII are successive cells of the lateral secondary appendage arising from the primary appendage. The lateral appendage extends beyond the base of the perithecium (arising from cell VII). Depending on the genus, there may be a single perithecium or multiple ones. Genera included in the Euceratomycetaceae are *Cochliomyces*, *Colonomyces*, *Euceratomyces*, *Euzodiomyces*, and *Pseudoecteinomyces*.

Taxa in the genus *Euzodiomyces* are exceptional among Laboulbeniales in the construction of their primary receptacle, which is many-celled and parenchymatous (Tavares, 1985; Santamaría, 2003). Other than in *Euzodiomyces*, this feature is only present in the genera *Columnomyces*, *Kainomyces*, *Scepastocarpus*, and *Zodiomyces*, all of which are classified in the Laboulbeniaceae (Rossi *et al.*, 2016). All genera but one (*Tettigomyces*) in Ceratomycetaceae are associated with aquatic hosts, whereas those in Euceratomycetaceae have terrestrial hosts. Finally, the family Laboulbeniaceae is recognized by the tiers of perithecial outer wall cells, which are four or five in number and unequal in height. Although the genus *Zodiomyces* has perithecial outer wall cells that are arranged in eight tiers subequal in height, Tavares (1985) also placed this genus in Laboulbeniaceae, in its own subfamily Zodiomycetoideae. This is in stark contrast to Thaxter (1908) who had placed *Zodiomyces* in the suborder Ceratomycetineae.

Currently, the classification system of Tavares (1985) is still in use, although molecular phylogenetic studies have repeatedly shown that several taxa within this system are polyphyletic. At least three subtribes, two tribes, and two subfamilies are polyphyletic (Goldmann and Weir, 2018; Haelewaters, 2018). In addition, *Herpomyces* was recently removed from the Laboulbeniales and placed in its own order with strong support from multiple sources (Haelewaters *et al.*, 2019d; Blackwell *et al.*, 2020). In the SSU rDNA phylogeny of Haelewaters (2018), four genera with species that are associated with aquatic hosts were placed basally in the Laboulbeniales order. This might be seen as evidence for an ecological viewpoint rather than, or in addition to, a structural-based one. All in all, while the classification of the Laboulbeniales order is in urgent need of complete revision, current taxon and character sampling is far too insufficient to make changes that are taxonomically stable. Continued development of molecular

protocols – optimized for scarce material, e.g., including a whole-genome amplification step prior to PCR – will lead to the progress needed to propose a stable evolutionary hypothesis for the order.

Notes on ecology

Where Laboulbeniales receive their nutrients from is not entirely understood, which can be partly explained by the presence of taxa without a penetrating haustorium (Tragust *et al.*, 2016). In addition, to date, attempts to obtain axenic cultures of Laboulbeniales have failed whereas transmission experiments of Laboulbeniales among hosts have been used to prove specificity and host-related nutritional requirements. Several authors put forward different hypotheses about nutrient uptake—including chitin of the integument, secretions from exocrine glands, substances available at the cuticle (waxy substances, components from plants, substrate, microbiota, host fecal materials), and waxy lipids produced by the epidermal cells. It is most widely accepted that nutrition is obtained through the attachment area. Support for this hypothesis came from Scheloske (1969) who injected Nile sulfate dye into an insect and observed it flowing from elytral tissues to *Laboulbenia* thalli.

Since Laboulbeniales are phenotypically plastic, their morphology can vary dramatically depending on the position on the host's integument as well as the sex of the host. Some authors considered these morphologies as separate taxa (Thaxter, 1926; Benjamin and Shanor, 1952; Benjamin, 1967), whereas others described them as morphotypes of the same biological species (Rossi and Kotrba, 2004; Rossi, 2006; Rossi and Proaño Castro, 2009; Santamaría and Faille, 2009; Haelewaters and Rossi, 2017). Based on molecular analysis, the phenomenon of position specificity was recently confirmed for Laboulbeniales from aquatic hosts (Goldmann and Weir, 2012); speciation was not connected to nutrition of the fungus, but to a highly specific and precise transmission of spores during copulation. However, the understanding of how Laboulbeniales from terrestrial hosts are directly transmitted, mainly by copulation (Scheloske, 1969), made an end to position specificity and sex-of-host specificity.

The need for a living host is clear, but the occasionally reported dramatic host shifts have remained unexplained for a very long time. Examples are *Rickia wasmannii* in ant nests parasitizing both *Myrmica* ants (main host) and co-habitating arthropods, which belonged to a different order (Diptera) and even subphylum (Chelicerata) (Pfliegler *et al.*, 2016), and *Stichomyces conosomatis* in a subterranean cave on *Speonemadus algarvensis* (Leiodidae), which represented the only report of this fungus on a host other than species of *Sepedophilus* (Staphylinidae) (Reboleira *et al.* 2017). Scheloske (1969) and later Majewski (1994) and De Kesel (1997) showed that a single species of *Laboulbenia* can have main, occasional, and accidental hosts. In most cases, these hosts occupy the same habitat. Based on his observations, Scheloske (1969) stated that plurivorous Laboulbeniales need both the host and its habitat and launched the concept of "ecological specificity" – although the term "habitat specificity" is more accurate because host specificity also assumes resource availability and niche specialization.

De Kesel (1996) carried out experiments testing the impact of habitat on growth, development, and transmission. Via a gradient analysis it was found that under certain habitat conditions, *L. slackensis* can be transmitted and will develop successfully on a series of atypical hosts. The potential host range of Laboulbeniales is much wider than initially thought and opportunities for host shifting are therefore entirely governed by the host, its behavior (habitat choice), and life history. Although host shifting of Laboulbeniales is possible under certain conditions, there are some barriers. Species of Laboulbeniales are fairly isolated on their respective host populations. This is because direct transmission of ascospores is mostly intraspecific (among hosts of the same species), in the form of grooming, copulation, and random aggregation contacts (*Herpomyces*: Richards and Smith, 1955; *Hesperomyces*: Nalepa and Weir, 2007; *Laboulbenia*: De Kesel, 1995a,b; *Rickia*: Haelewaters *et al.*, 2015a). Indirect transmission is negligible because of the short lifespan of ascospores (De Kesel, 1995b; Cottrell and Riddick, 2012), and opportunities for interspecific contacts with other hosts are often few because of their separation in time and space—unless they cohabit the same microhabitats.

Most species of Laboulbeniales only develop on adults; transmission can only take place if adult generations overlap. Because of this, suitable hosts that are parasitized by a given species of Laboulbeniales in warmer areas lack the parasite in colder localities (northern, alpine), where they overwinter as larvae (Huldén, 1983). Parasite prevalence, that is, the number of parasitized host specimens in a population, fluctuates greatly throughout the year and is affected by the host life cycle (spring breeders vs. autumn breeders), the emergence period of new generations (Scheloske, 1969), and host population density (De Kesel, 1995a). Thallus density, the number of thalli on a host individual, on the other hand, seems to increase with increasing age of the host (Haelewaters *et al.*, 2015a; De Kesel *et al.*, 2016). This is an important feature because it boosts opportunities for transmission of ascospores between old and new generations.

Thallus morphology

The *thallus* (**Fig. 3**) is a multicellular unit with a restricted number of cells, derived from two-celled ascospores through a defined number of mitotic divisions in multiple planes (Blackwell *et al.*, 2020). A primary septum separates the larger cell of the ascospore from the smaller one. This septum is often visible by its thickness and color, even in mature thalli. The main axis of the thallus is formed by the receptacle, which is the part of the multicellular unit that is connected to the host by means of a foot. The receptacle and foot are derived from the larger cell of the ascospore, which is released first from the perithecium. Additional divisions of particular cells of the receptacle produce the perithecium or perithecia. The perithecium is the only spore-forming structure of the Laboulbeniales; there are no asexual spores. The smaller cell of the ascospore produces the primary appendage system, which carries the spermatia-producing antheridia. The entire ontogeny, from ascospore to mature thallus, was studied for a few *Laboulbenia* species (Tavares, 1985, De Kesel, 1989). In terms of orientation, the anterior side is the one on which the perithecium

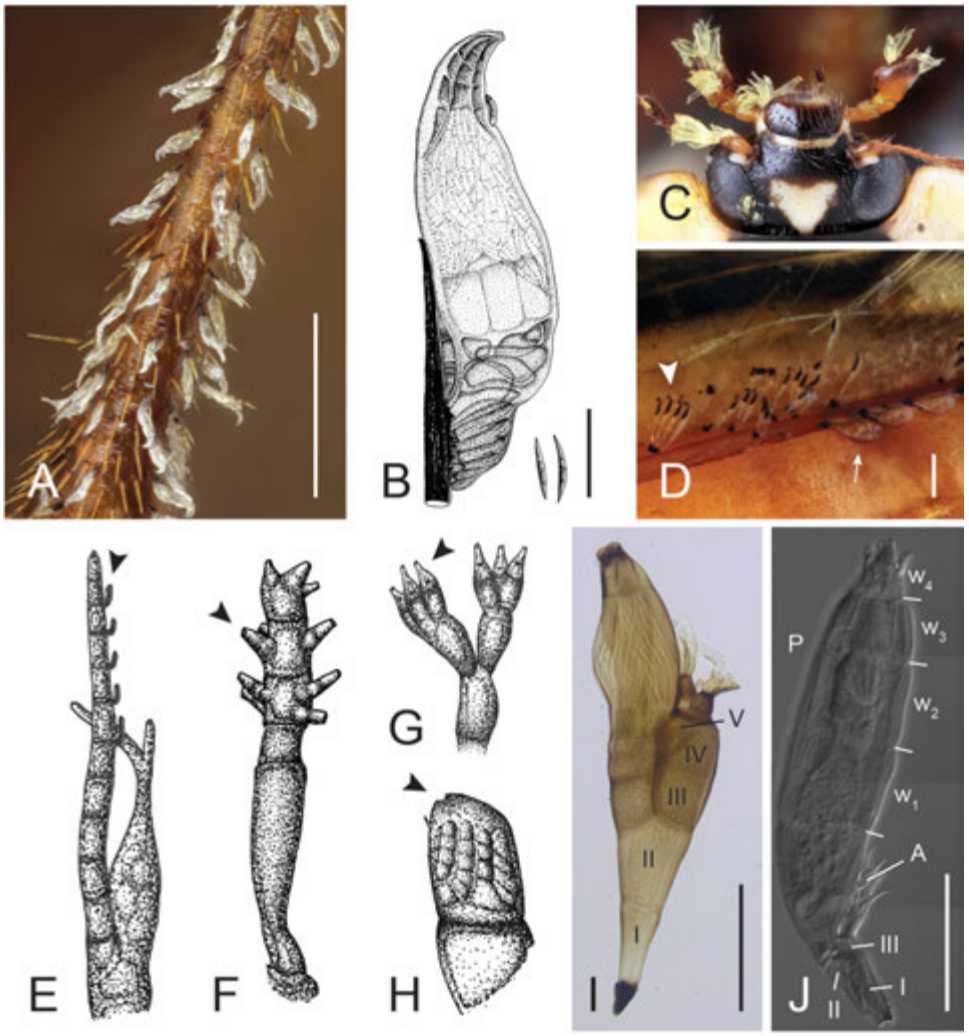

Fig. 3 Herpomycetales and Laboulbeniales. **A–B.** *Herpomyces chaetophilus* on *Periplaneta americana*. **A.** Thalli attached to the setae of an antenna. **B.** Single thallus of *H. chaetophilus* attached to an antennal seta. Pencil drawing by Jingyu Liu. **C**. A *Harmonia axyridis* ladybird with tufts of *Hesperomyces* thalli on its mouthparts and left eye. **D.** Growth positions of *Chitonomyces* thalli on the left elytral margin of *Laccophilus hyalinus*; thalli of *C. melanurus* (arrowhead) are found above and on the epipleuron, whereas *C. paradoxus* thalli (arrow) consistently grow on the lower part of the margin. **E–H.** Spermatia-producing structures. Pencil drawings by Jingyu Liu. **E.** Appendage cells forming spermatia exogeneously (arrowhead) in *Drepanomyces* sp. (Ceratomycetaceae). **F.** Simple antheridia (arrowhead) in *Arthrorhynchus nycteribiae* (Laboulbeniaceae). **G.** Tufts of simple antheridia (arrowhead) in *Laboulbenia disenochi* (Laboulbeniaceae). **H.** Compound antheridium, with spermatia leaving the chamber through a single opening (arrowhead), in *Neohaplomyces medonalis* (Peyritschiellaceae). **I.** Mature thallus of *Laboulbenia fuscata* from *Pterostichus* sp. collected in Argentina; the receptacle is formed by cells I through V (labeled); cells III, IV, and V in *Laboulbenia* are often referred to as the androstichum. From the Roland Thaxter collection of slide mounts at the Farlow Herbarium. **J.** Mature thallus of *Hesperomyces virescens* (sensu lato) from *Harmonia octomaculata* collected in Micronesia; annotated are cells I, II, and III of the receptacle, the appendage (A) with simple antheridia, and the perithecium (P) with four tiers of wall cells (w_1 to w_4). Scale bars A 500 μm; B, D 50 μm; I, J 100 μm.

is located, whereas the posterior side is the side away from the perithecium. Other authors use ventral and dorsal for anterior and posterior, respectively.

Receptacle

The primary receptacle forms the base for all parts of the thallus. Its shape and structure are extremely variable within the order, and this variability is an important criterion in generic delimitation. Apparently, the lower cell of the ascospore generally divides into 3 cells denoted by Roman numerals I, II, and III. Further divisions in different planes may take place, depending on the genus. Many genera, those in the subtribe Stigmatomycetinae, have only those three cells in the receptacle but their positions are variable with respect to one another. Cell I is the basal cell, forming the connection with the host's integument (referred to as the foot). Multiple divisions of cell I can occur, for example in female thalli of *Dimeromyces*. These secondary cells will further give rise to

perithecia or sterile appendages. Cell II, the suprabasal cell, generates the perithecium by successive divisions. Cell II undergoes multiple divisions in many genera, forming an elongate uniseriate receptacle. Examples of genera with this structure are *Chaetomyces*, *Ecteinomyces*, *Filariomyces*, and *Ormomyces*. Secondary divisions of cell III can occur. For example, in the genus *Laboulbenia*, these divisions form cells IV and V. The entire complex of cells III to V is called androstichum (**Fig. 3I**). Some species of *Laboulbenia* have an undivided cell III + IV or cell III + IV + V (e.g., *L. nisotrae*, *L. obesa*, *L. richardiana*).

Perithecium

The perithecium is derived from the receptacle, in species without secondarily divided receptacle cells it arises from divisions of cell II. Benjamin (1971) described three types of perithecial development. In the first type, a single cell arises laterally from the receptacle to divide into a lower and upper cell. The lower cell, by continued divisions, gives rise to the perithecial stalk cell (VI), secondary stalk cell (VII), and basal cells m, n, and n′. The upper cell will give rise to the female sexual organ, which initially comprises three cells: basal carpogenic cell, trichophoric cell, and terminal trichogyne. The trichogyne is a thin appendage-like outgrowth of the young perithecium. It may or may not develop into a multicellular simple or branched structure, depending on the species. Its function is to receive spermatia. Before the perithecium is mature, the trichogyne will deteriorate, often leaving a visible scar. This "carpogonial upgrowth" is enveloped by the perithecial walls, which arise from cells m (forming a single vertical row of wall cells) and n and n′ (forming three rows).

After interception by the trichogyne, the male nucleus from a spermatium will migrate to the carpogenic cell, thus resulting in the formation of an ascogenous cell (or multiple ones by mitotic divisions), in which both the male and female nuclei are present. This is the dikaryotic phase of the Laboulbeniales life cycle. Asci are produced by mitotic divisions of the ascogenous cells in multiple planes. Upon fusion of the two nuclei, the diploid ascus mother cell is formed, which after meiosis gives rise to an ascus with 4 ascospores. This developmental type was described and illustrated by Thaxter (1896) for *Laboulbenia elongata*, *Peyritschiella geminata*, and *Stigmatomyces baeri*. According to Benjamin (1971) there is only one genus of Laboulbeniales that does not follow this type of development; *Coreomyces* forms what Thaxter (1908) named a pseudoperithecium.

The mature perithecium is more or less elongated and narrowed towards the tip (distally). Sometimes there is a clear differentiation into a rounded or ovoidal venter and a narrow neck, terminating in an ostiole. The perithecial wall cells surrounding the ostiole often form distinct lips (e.g., in *Hesperomyces*) or (sub)apical outgrowths (e.g., in *Diphymyces*). The perithecium usually consists of a well-defined number of cells. The perithecial wall cells appear in two layers; the external wall cells are clearly visible and have taxonomic importance (Tavares, 1985; Majewski, 1994). The most ancestral perithecium is the one in which each of the four vertical rows of outer wall cells consists of many cells that are equal in height, as in Ceratomycetaceae. This was stated by Tavares (1985) and supported by the use of sequence data (Goldmann and Weir, 2018; Haelewaters, 2018). Morphological studies of the genera *Nycteromyces* and *Polyandromyces* (Dimorphomyceteae) failed to distinguish perithecial cell walls (Thaxter, 1920, 1924; Haelewaters, 2018). Presumably this represents a highly derived situation (Tavares, 1985).

Appendage and antheridia

The primary appendage usually is a direct continuation of the receptacle axis. It is produced by divisions of the upper, smaller cell of the ascospore. In some genera, the primary appendage is very simple, consisting of one or two cells only. Examples are *Dioicomyces* and *Filariomyces*. In very few species the appendage can even become aborted (Tavares, 1985). Well-developed primary appendage systems exist in many species of, e.g., *Corethromyces* and *Laboulbenia*. Sometimes, the original spore apex remains visible at maturity as a spinose process because the branches are formed at a level below the apex. This process is an important feature to identify species in the genera *Acompsomyces*, *Eucantharomyces*, *Ilyomyces*, and *Rhachomyces* (Santamaría, 2003, 2006; Haelewaters, 2013; Santamaría *et al.*, 2020a). The primary appendage system of *Laboulbenia* deserves extra attention. Its basal cell, called insertion cell or cell e, is flattened and usually obscure and carries the inner and outer appendages. The inner appendage bears flask-shaped, simple antheridia. The outer appendage is usually longer, simple or branched, and almost always sterile.

The primary appendages of *Chitonomyces* and *Hydraeomyces* break off early, right above the constricted black septum (Tavares, 1985). The primary appendages of *Columnomyces* and *Diphymyces* are usually partly or completely broken off (Thaxter, 1918, 1931; Benjamin, 1955; Haelewaters *et al.*, 2014; De Kesel and Haelewaters, 2019; Perreau *et al.*, 2021). This damage has been linked to the behavior of the host insects, Cholevinae (Coleoptera, Leiodidae). Cholevine beetles have evolved a largely underground lifestyle and make extensive use of narrow channels and tunnels in the soil, which may account for breakage of parts of Laboulbeniales thalli on these hosts (Sokolowski, 1942). Also the extensive appendage system of *Laboulbenia clivinalis* regularly breaks off (and regenerates, Majewski, 1994). Similar to cholevines, *Clivina fossor*, the host of *L. clivinalis*, has a partly subterranean lifestyle (De Kesel, 1995a).

When sterile or antheridial branches are derived from the lower cell of the ascospore, they are referred to as secondary appendages. All appendages of *Scepastocarpus* and *Zodiomyces* are secondary in origin. Little is known about the function of sterile appendages, whether primary or secondary. Cavara (1899) speculated that thalli could retrieve nutrients from the environment by means of their sterile appendages. De Kesel (1996) showed experimentally that the successful establishment of *Laboulbenia slackensis* requires not only a suitable host but also favorable environmental conditions, which could be linked to the extensive appendage system of that species. Recently, Tragust *et al.* (2016) found no visible penetration damage at the host integument using light and electron microscopy techniques in four species of Laboulbeniales, revealing the necessity for alternative explanations to the hypothesis that Laboulbeniales may only receive nutrients through a haustorium. Further experimental work might be directed

toward the function of the sterile appendages; this would entain injecting dye in thalli of both haustorial and non-haustorial representatives of Laboulbeniales (see *Notes on ecology*).

Spermatia are produced either exogeneously or endogeneously within simple or compound antheridia (**Fig. 3E–H**). Exogenous spermatial formation has mainly been observed in species that have aquatic hosts (Weir and Blackwell, 2005), such as species of *Ceratomyces* and *Zodiomyces*. In these genera, spermatia may be borne on intercalary cells or terminally on a short branchlet (Majewski, 1994). Simple antheridia are flask-shaped, with the neck serving as a discharge tube. Sometimes, old antheridia can proliferate into sterile branches, this is often seen in members of *Laboulbenia*. In some genera, corner cells or intercalary cells of the appendage serve as antheridia with only the discharge tube being free. Most Laboulbeniales possess simple antheridia. Compound antheridia only occur in taxa of Monoicomycetoideae and Peyritschielloideae. Antheridial cells are structurally united and release their spermatia into a chamber that has a single opening. In the subfamily Monoicomycetoideae, compound antheridia are distally rounded and lack a discharge tube. Compound antheridia with an elongated neck occur in the Peyritschielloideae. This observation led Faull (1911) to suggest that compound antheridia had arisen independently more than once. Almost a century later, this suggestion was confirmed based on molecular phylogenetic data (Goldmann and Weir, 2018; Haelewaters, 2018). Antheridial characters were important to Thaxter's (1896, 1908) – obsolete – classification system.

Ascospores

The ascospores of Laboulbeniales are two-celled, hyaline, elongate, and spindle-shaped. They are typically surrounded by a mucilaginous envelope, which provides adhesiveness. In addition, the foot is usually melanized before release. The ascospores are almost exclusively transferred by the activities of the host (De Kesel, 1995a; Cottrell and Riddick, 2012). Ascospores are produced in perithecia such that their larger cell, which becomes attached to the host, is directed upwards and subsequently released first.

Herpomycetales

Until very recently, discussions of "Laboulbeniales" included the order Herpomycetales, which is now separated based on molecular and morphological evidence. Herpomycetales was erected following the molecular phylogenic analysis of a three-locus rDNA dataset. The order is monotypic, with the dioecious *Herpomyces* as the single genus. The genus currently includes 27 species, all described between 1902 and 1931 except for two recent species, *H. shelfordellae* and *H. spegazzinii* (Haelewaters *et al.*, 2019d; Gutierrez *et al.*, 2020). All species are exclusively associated with cockroaches (Blattodea), both nymphs and adults. Thalli of *Herpomyces* are developmentally and morphologically distinct (Tavares, 1965, 1966, 1980, 1985; Hill, 1977). This evidence provides additional support for the idea that thallus formation in the two orders Herpomycetales and Laboulbeniales has evolved independently (**Fig. 1D**). For example, whereas both orders have double-layered perithecia, the way their perithecial walls are formed is fundamentally different. Ascus formation, number of ascospores per ascus, and positioning of the ascospore septum are also different between Herpomycetales and Laboulbeniales (reviewed in Haelewaters *et al.*, 2019d). *Herpomyces* also perforates its host in multiple places whereas Laboulbeniales perforate their host only at the single point of attachment or not at all (Tragust *et al.*, 2016). Species of *Herpomyces* can vary in their morphology depending on their position on the host (Thaxter, 1908, Tavares, 1985), as is also the case with some members of Laboulbeniales.

The thallus of *Herpomyces* has a differently structured receptacle compared to genera of Laboulbeniales. The primary receptacle of female thalli is small, typically consisting of four cells. The suprabasal cell gives rise to a secondary axis that consists of a series of narrow cells each perforating the integument of the host by small haustoria. Male thalli are similar in that they have a primary axis, usually consisting of four superposed cells, and that the suprabasal cell may produce a secondary axis; both the third and fourth cell may give rise to a single cell or branch carrying antheridia. The perithecium of Herpomycetales has four vertical rows of outer wall cells each consisting of many cells equal in height. This condition, which is also seen in Ceratomycetaceae, has been considered the "ancestral" perithecium but the situation is likely more complex provided the thallus of Herpomycetales and Laboulbeniales may have evolved independently. This evolutionary hypothesis is supported by the development of the perithecium that is different in *Herpomyces* and Laboulbeniales. The entire perithecium of *Herpomyces* develops from an outgrowth of the suprabasal cell of the 4-celled primary receptacle, by subsequent transverse and longitudinal divisions (Tavares, 1965, 1966). The carpogonial upgrowth(s) is initiated by a specific outer wall cell. Finally, in the *Herpomyces* ascus mother cell, mitosis will take place after meiosis, forming an ascus with 8 ascospores, as do most of the other species in Ascomycota. The entire ontogeny, from ascospore to mature thallus, was studied for *Herpomyces ectobiae* (Tavares, 1985).

Contrary to infections with most species of Laboulbeniales, *Herpomyces* infections often display a high parasite prevalence. For example, Richards and Smith (1955) mentioned a 100% prevalence of *H. stylopygae* on 50 *Blatta orientalis* cockroaches. Similarly, Wang *et al.* (2016) reported a 96.8% prevalence of *H. chaetophilus* on 31 *P. americana* roaches. This can be explained by the fact that populations of cockroaches are often densely packed, they occur in moist, damp environments, and they are in constant contact with each other, for example by their grooming behavior. Pfliegler *et al.* (2018) studied eleven populations of cockroaches, originating from either pet stores, biological supply companies, or laboratory colonies. In eight populations, infections with *Herpomyces* spp. were detected, and parasite prevalence ranged between 8.77% and 86.36%. The authors suggested that at least some species of *Herpomyces* are spread by globally invasive host species as well as through the international pet and pet food trade.

Pyxidiophorales, Hyphal Mycoparasites

The order Pyxidiophorales contains the perithecial genus *Pyxidiophora* and *Mycorhynchidium*, reported to vary by its cleistothecial form. *Pyxidiophora* and close relatives are contact mycoparasites varying in their reliance on a host fungus from greatly improved growth to obligate fungal biotrophy (Kirschner, 2003; Jacobs *et al.*, 2005). The life cycle of most species of *Pyxidiophora* is complicated, consisting of three different morphs: (1) a dispersal morph (*Thaxteriola* state) derived from an ascospore that delivers conidia to a fresh substrate, (2) a conidial morph developed on hyphae, and (3) an ascospore-producing perithecial morph to come full circle (**Fig. 5**). Only 21 species of *Pyxidiophora* have been formally described although it is unclear whether they are all distinct (Lundqvist, 1980; Doveri and Coué, 2006). At least five undescribed species are known (M. Blackwell and M. Gorczak, unpublished) and more are certain to be discovered. Names listed as synonyms of *Pyxidiophora* in MycoBank (2020) are based on all three life cycle states of *Pyxidiophora* (see below).

Taxonomy and Phylogeny

Pyxidiophora was described by Brefeld and Von Tavel (1891), and its complicated nomenclatural history discussed by Lundqvist (1980) resulted in typification with *Pyxidiophora asterophora*. Both MycoBank (2020) and Index Fungorum (2020), however, list *P. nyctalidis* as the type species, likely because of recent retroactive changes in the *International Code of Nomenclature for algae, fungi, and plants* (Turland *et al.*, 2018). The gradual maturation of the perithecium and its ephemeral nature, slow ascospore maturation, difficulty of cultivation, and the complex three-morph life history of these fungi, hampered the use of morphological characters. For example, the early deliquescent asci lead some researchers to describe *Pyxidiophora* perithecia as pycnidia (e.g., *Mycorhynchus)* (Petch, 1936). These problems have caused creation of many synonyms based on the three different morphs (perithecial, hyphal phialidic conidial, and ascospore-derived dispersal morphs) in the life cycle.

Genera based on the perithecial morph are considered synonyms of *Pyxidiophora*, often based on immature specimens: *Ascolanthanus*, *Coprophilous*, *Mycorhynchus*, *Rhynchonectria*, *Treleasia* (Hawksworth and Webster, 1977; Lundqvist, 1980), and perhaps the cleistothecial *Mycorhynchidium*. The type of *Treleasia* is no longer intact, but Spegazzini's drawing of the ascospores and perithecial neck (Arambarri *et al.*, 2007) leave no doubt of the identity of a species from an unusual habitat: rolled, deteriorating leaves of sugar cane. Named phialidic conidial morphs derived from hyphae include *Chalara*-like, *Gabarnaudia*-like, *Gliocephalis*, and *Pleurocatena* (Arnaud, 1952, 1953; Blackwell and Malloch, 1989b; Jacobs *et al.*, 2005; Gams and Arnold, 2007). No perithecial state is known for *Gliocephalis hyalina* (Jacobs *et al.* 2005), but an SSU rDNA sequence places it in *Pyxidiophora*, and culture attempts of this fungus were successful only when co-inoculated with *Fusarium* as expected of a mycoparasite (Corlett, 1986). Other synonyms are based on the dispersal morph developed from the ascospore: *Acariniola*, *Thaxteriola*, and perhaps other ascospore-derived forms (Blackwell, 1994). The presumptive ascospore derived morphs including *Amphoropsis*, *Endosporella*, *Entomocosma*, *Myriapodophila*, and *Thaxteriola* spp. were placed in an informal group, "Thaxteriolae," although this name has not been defined consistently (Thaxter, 1914, 1920; Spegazzini, 1918; Majewski and Wiśniewski, 1978; Blackwell, 1994; Blackwell *et al.*, 2020). Gäumann (in Gäumann and Dodge, 1928) "regarded" some of these forms as male thalli of Laboulbeniales, an idea rejected by Thaxter (Gäumann and Dodge, 1928).

The ordinal placement of *Pyxidiophora* was uncertain until DNA analyses became available. Most often it was placed in the order Hypocreales (e.g., Lundqvist, 1980; Barr, 1990) or considered a member of the order Ophiostomatales (von Arx and van der Walt, 1987; Eriksson and Hawksworth, 1989, Blackwell and Spatafora, 1994). Based on a primarily speculative review (Blackwell and Malloch, 1989b), Eriksson and Hawksworth (1993), placed *Pyxidiophora* in the Laboulbeniales. Soon after, with increased taxon sampling, the higher-level classification of Pyxidiophorales (P.F. Cannon in Kirk *et al.*, 2001) was formally established in the family Pyxidiophoraceae (Arnold, 1971). If they exist, closer relatives of the class Laboulbeniomycetes other than Sordariomycetes among Ascomycota have not been discovered (Blackwell *et al.*, 2020).

Morphology of Pyxidiophorales Life Cycle States

The three-morph life cycle of *Pyxidiophora* described above with its specialized dispersal morph, is unique among Ascomycota (**Fig. 5**). It has been compared to the life cycles of plant-parasitic rust fungi (Basidiomycota) with respect to Tranzschel's law in its host associations (Malloch, 1995; Blackwell *et al.*, 2020).

Perithecial morphs

Ascocarps of *Pyxidiophora* are perithecia (**Fig. 4C,D**). One species, *Mycorhynchidium saccatum*, was described as cleistothecial from a moist chamber development (Malloch and Cain, 1971). The pseudoparenchymatous perithecia usually have a distinct bulbous base with irregularly angular to globose cells and tapers slowly to an elongating neck composed of parallel, closely packed cells (Hawksworth and Webster, 1977; Lundqvist, 1980; Kirschner, 2003; Doveri and Coué, 2006). A unique feature of the mature perithecial peridium is that it is composed of a single layer of cells. This unusual character is known only in species of *Kathistes* among other perithecial ascomycetes (Malloch and Blackwell, 1990). Interascal tissues (e.g., paraphyses) have not been observed in any species of *Pyxidiophora*. The presence of an apical ring may be ephemeral and Lundqvist (1980) does not consider it a generic character. Perithecia can be single or grouped, free on the substrate or developed on a stroma, hairy or naked, and short to very long necked. Unlike thallus-forming Laboulbeniomycetes,

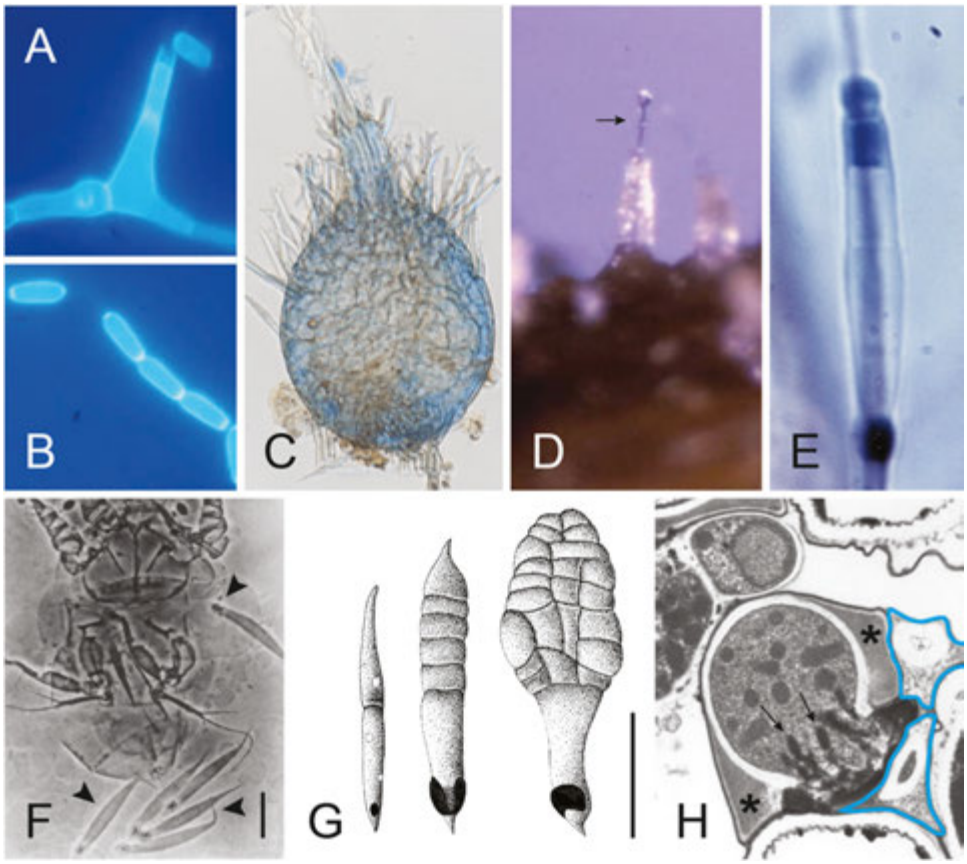

Fig. 4 Pyxidiophorales. **A–B.** *Chalara*-like conidial hyphal morph from a two-membered culture on Leonian's agar. Fluorescence microscopy using calcofluor white stain. **A.** Conidial succession and second conidium differentiated within a phialide. **B.** Chain of conidia showing contact pads; note the similarity with conidia of *Gliocephalis hyalina* (Jacobs *et al.*, 2005). **C.** Perithecium of *Pyxidiophora corallisetosa*, Białowieża Primeval Forest, Poland. **D.** Perithecium of *Pyxidiophora* sp. in moist chamber of moose dung, New Brunswick, Canada; the base is partially embedded in the dung; ascospores at the perithecial tip (arrow) are ready to attach to a disperser. **E.** Ascospore-derived conidial morph (*Thaxteriola*) with darkened attachment region, developed in moose dung moist chamber, Ontario, Canada. Lactophenol-cotton blue stain. **F.** *Tarsonemus ips* mite with six *Thaxteriola* sp. thalli (arrowheads). Picture by John C. Moser. **G.** Developing thallus of *Thaxteriola* sp. The parenchymatous thalli are not common until after the mites are held in moist chambers past the time they would become phoretic. Pencil drawing after David Malloch by Jingyu Liu. **H.** Attachment region of the ascospore-derived conidial morph; note the thickened spore wall (*) and secretory channels (arrows) with electron dense material that are similar to those in *Coreomycetopsis* and *Laboulbeniopsis*. Basal cells of other ascospores are encircled in blue. Transmission electron micrograph. **D–F, H.** Reprinted with permission from Mycologia. © The Mycological Society of America. Scale bars F, G 25 μm. Reproduced from Blackwell, M., 1994. Minute mycological mysteries: The influence of arthropods on the lives of fungi. Mycologia 86 (1), 1–17. Blackwell, M., Perry, T.J., Bridges, J.R., Moser, J.C., 1986. A new species of *Pyxidiophora* and its *Thaxteriola* anamorph. Mycologia 78 (4), 605–612.

cell-by-cell development of fruiting bodies of Pyxidiophorales is not known in detail but likely is not fixed as in Herpomycetales and Laboulbeniales. Asci are not reported for all species because of their early evanescence (Hawksworth and Webster, 1977; Lundqvist, 1980). Production of ascocarps in pure culture was reported for *Pleurocatena acicularis* (Gams and Arnold, 2007) and in mixed cultures for *Pyxidiophora* [as *Ascolanthanus*] *trisporus* (Cailleux, 1967), *Pyxidiophora asterophora* (Brefeld and Von Tavel, 1891), *P. corallisetosa* (Kirschner, 2003), and others (M. Blackwell, unpublished).

Asci of *Pyxidiophora* are unitunicate, thin-walled, non-amyloid and fusiform, clavate or obovoid. The number of ascospores per ascus is 3–8. The common condition of three ascospores per ascus in this genus is unusual among ascomycetes. Viewed with transmission electron microscopy, 3-spored asci appear to be due to exclusion of one of four post-meiotic nuclei from the enveloping membrane system during ascosporogenesis (M. Blackwell and E.A. Richardson, unpublished); details are not known. Asci mature sequentially and the ascospores are released passively in a sticky droplet hanging at the tip of the perithecium, awaiting an arthropod for dispersal (Breton and Faurel, 1967; Blackwell, 1994). Ascospores are two-celled, single-septate, usually symmetrical, and enveloped in a mucilaginous sheath before maturity (Lundqvist, 1980; Blackwell and Malloch, 1989a; Blackwell *et al.*, 1989). At maturity, ascospores of *Pyxidiophora* are single-septate, elongated, and fusiform to subclavate. For all species that have been observed at maturity, ascospores have a darkened attachment apparatus at their exiting ends by the time they exit the

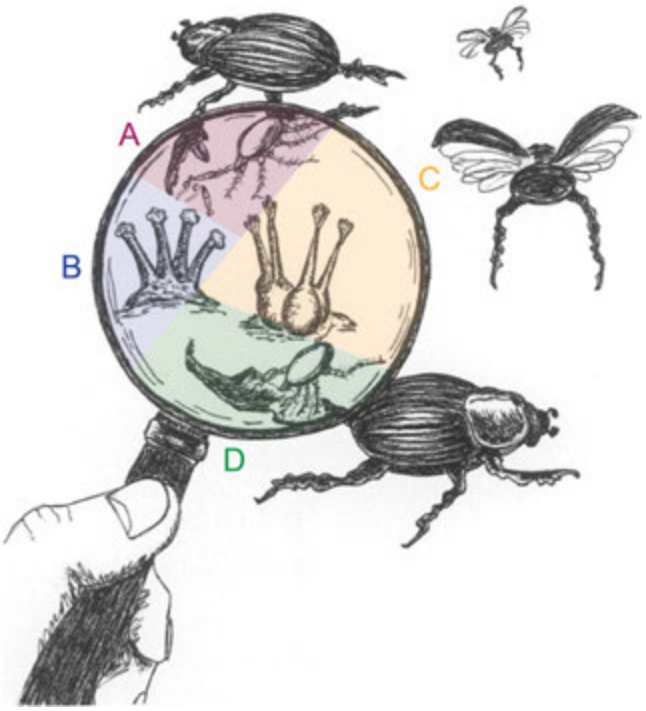

Fig. 5 Cartoon illustrating the life history of *Pyxidiophora* sp. **A.** A beetle and phoretic mite carrying an ascospore-derived dispersal morph arrive at a substrate where a suitable host fungus is growing. **B.** Conidial morph develops nourished by the host fungus in the substrate and produces conidia, which are spread locally on the surface of the dung by immature mites and nematodes. **C.** The next event is the development of perithecia, followed by evanescence of the asci and release of sticky ascospores to the tip of the perithecium neck. **D.** The phoretic mite bearing ascospores attaches to a beetle disperser. The attached ascospores develop into the dispersal morphs to reinitiate the cycle in another targeted substrate. Pencil drawing by Ty Keller. Reprinted with permission from Mycologia. © The Mycological Society of America. Reproduced from Blackwell, M., 1994. Minute mycological mysteries: The influence of arthropods on the lives of fungi. Mycologia 86 (1), 1–17.

perithecium. This is one of the inconsistent features in species descriptions, resulting from the observation of immature material (Blackwell, 1994).

Phialidic conidial hyphal morphs

A number of different conidial states arise from the hyphae, and these were described in connection with *Pyxidiophora* (Lundqvist, 1980; Gams and Arnold, 2007). The conidia of *Pyxidiophora* are blunt-ended or bullet-shaped conidia, often in chains. Arthric anamorphs probably were inaccurately mistaken for chains of conidia (Cailleux, 1967). The holoblastic conidia are produced in phialides on the mycelium. Most have been described as *Chalara*-like (e.g., in *P. asterophora*, *P. spinuliformis*) or *Gabarnaudia*-like (e.g., in *P. corallisetosa*, *P. cuniculicola*). *Pleurocatena*, first observed in the early 1950s (Arnaud, 1952, 1953; Arambarri *et al.*, 1981), was studied again in culture by Gams and Arnold (2007) with the new strains described as *Gabardnaudia*-like, although living cultures were not maintained. The authors distinguished the conidial state on the presence of setae mixed among the phialides. They connected the conidial state to *Pyxidiophora* sp. when perithecia were developed in a culture of *Pleurocatena acicularis* after several months. Other hyphal forms, probably also with phialidic conidia have been described, including *Gliocephalis hyalina* for which no perithecial state is known (Jacobs *et al.*, 2005). *Pyxidiophora spinulorostrata* was described as having heavily walled outgrowths near the perithecial necks that produced conidia (Webster and Hawksworth, 1986). Perithecia of the same species from the type locality (River Teign, Devon), however, did not produce such conidiophores; instead, a *Gabarnaudia*-like anamorph was observed before appearance of perithecia (M. Blackwell, unpublished).

Ascospore-derived conidial morphs

The second type of conidial morph in the three-morph life cycle develops from an ascospore (**Fig. 4E–H**). Although the entire life cycle has not been studied in all species, the ascospore-derived form occurs consistently in the best studied species of *Pyxidiophora*. Germination by germ tube has been observed on agar but rarely. Most of the ascospore-developed morphs have been referred to as *Thaxteriola* or *Acariniola*. The ascospores have gelatinous mucus sheaths early in ascospore maturation. The dark-pigmented attachment apparatus develops at the exiting end of the ascospores by the time they are released in a sticky droplet at the tip of the perithecial until disperser contact. In at least five observed species of *Pyxidiophora* the primary dispersers were phoretic mites. TEM revealed the basal cell apparatus had a system of channels with an electron-dense material that were proposed to secrete a presumptive "glue" to attach the cell to the phoretic mite (**Fig. 4H**; Blackwell, 1994). After attachment of an ascospore to the

phoretic mite disperser, conidium development began (**Fig. 4E**). Ascospores of one strain were examined for up to a week under glass cover slips on agar. Yeast cells budded from the conidia, and within 24 h, several rounds of yeast cells had been produced and the yeast cells developed germ tubes several days later (Blackwell and Malloch, 1989b). Unless a fungal host was available, there was no further development in that strain.

As mentioned above, a number of minute fungi described from insects have been suggested to be conidial morphs of *Pyxidiophora* (Blackwell, 1994). The genera *Amphoropsis*, *Endosporella*, *Entomocosma*, *Myriapodophila*, and *Endosporella*, were described by Spegazzini (1918) and Thaxter (1920). Spegazzini suggested grouping a number of these minute fungi in the "Thaxteriolae" but Thaxter (1920: 15) doubted whether "these uninteresting little plants" were closely related to each other (see Blackwell *et al.*, 1986b, 2020; Blackwell, 1994). After *Acariniola* was described (Majewski and Wiśniewski, 1978), Lundqvist (1980) placed that genus in synonomy with *Pyxidiophora*.

Fine-Tuned Life Histories

The life history of *Pyxidiophora* spp. is a fine-tuned association of a variety of organisms. Accumulating evidence from axenic culture attempts has led to the conclusion that most species of *Pyxidiophora* are mycoparasitic or at least they grow better in two-membered culture with appropriate fungi (Corlett, 1986; Webster and Hawksworth, 1986; Blackwell and Malloch, 1989b; Kirschner, 2003; Jacobs *et al.*, 2005). Some species of *Pyxidiophora* were once considered saprotrophs (Lundqvist, 1980), and difficulties in obtaining axenic cultures led to earlier assumptions of nematophagous or bacteria-dependent modes of nutrition (Cailleux, 1967). The superb light and transmission electron micrographs of *Gliocephalis hyalina* (Jacobs *et al.*, 2005) provided strong evidence of contact mycoparasitism. It should be noted that two strains of *Pyxidiophora arvernensis* (CBS 657.82, CBS 253.81) isolated from *Rhizoctonia solani* baits in soil, have been used for DNA sequencing, because they do grow, albeit poorly, in axenic culture (Schoch *et al.*, 2012).

Timing is essential to the closely associated assemblage of organisms. For example, species on dung substrates use fungal hosts that arrive with the dung substrate by gut passage (Blackwell and Malloch, 1989b). Potential fungal hosts for *Pyxidiophora* spp. on seaweed probably arrive on the beach already growing in the seaweed, ready to nourish *Pyxidiophora* conidial morphs dispersed by arthropods on the beach (M. Blackwell and D. Malloch, unpublished). Kirschner (2003) pointed out that *P. corallisetosa* and *P. cuniculicola* and their fungal hosts were dispersed together by arthropods. *Pleurocatena acicularis*, a *Pyxidiophora* hyphal conidial state, was baited with fungi from soil (Gams and Arnold, 2007). The actual host of *P. asterophora*, observed on the mushroom of *Asterophora lycoperdoides* (Agaricales, Agaricomycetes), is unclear. *Asterophora* spp. are parasites of other mushrooms (*Lactarius* and *Russula* species), and an assemblage of organisms is present on the *Asterophora* mushrooms, including a tremellaceous yeast (Prillinger *et al.*, 2007) and fungi and bacteria developed in age (M. Blackwell, unpublished). The actual host of *Pyxidiophora asterophora*, therefore, remains unknown, but it may not be the mushroom. Buller (1924) suggested air dispersal of *Asterophora* chlamydospores and, although scarce in some of the mushrooms, basidiospores. Because the timing of arrival and dispersal is essential in the life history, it would be informative to know if these events occur consistently.

Documented fungal hosts used by *Pyxidiophora* include *Ascobolus* sp. (Pezizales, Pezizomycetes) growing on moose dung for *Pyxidiophora* sp. (**Fig. 6A**; Haelewaters, 2014), *Clonostachys rosea* (Hypocreales) for *P. corallisetosa*, *Esteya vermicola* (Pezizomycotina *incertae sedis*) for *P. cuniculicola* (Kirschner, 2003), *Fusarium poae* (Hypocreales) on seeds of *Triticum aestivum* for *P. lundqvistii* (Corlett, 1986), *Neonectria lugdunensis* (Hypocreales, Sordariomycetes) for *Pyxidiophora spinulorostrata* (Webster and

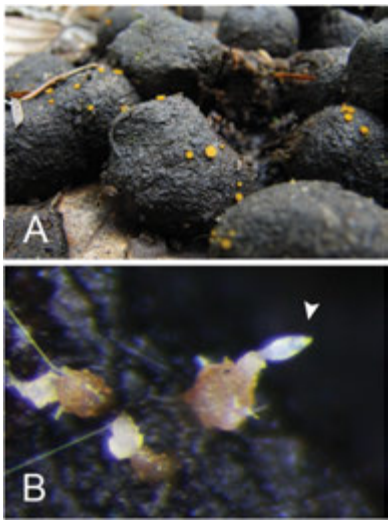

Fig. 6 Substrates for *Pyxidiophora*. **A.** Moose dung with apothecia of *Ascobolus* sp., from which perithecia of *Pyxidiophora* were isolated (White Mountain National Forest, Maine, USA). **B.** Perithecium of *P. corallisetosa* with package of ascospores at the tip (arrowhead), from a gallery of *Ips typographus*, the European spruce bark beetle, in Norway spruce (Białowieża Primeval Forest, Poland).

Hawksworth, 1986), and apothecia of coprophilous Pezizales for *Pyxidiophora* sp. and *P. spinuliformis* (Blackwell and Malloch, 1989b). Specificity of the fungal hosts of *Pyxidiophora* has not been studied in detail but has been noted (Corlett, 1986; M. Blackwell and D. Malloch, unpublished).

Blackwell and Malloch (1989b) studied the life histories of *Pyxidiophora* sp. and *Pyxidiophora spinuliformis* from moose dung in Algonquin Park, Ontario, Canada, and gathered data on timing of those species. The study was conducted by leaving the major part of a dung pile in the field for daily comparison with portions of the same substrate in moist chamber in the laboratory. On the day of deposition, the dung had no surface growth noticeable to the eye. The first appearance of *Pyxidiophora* sp. occurred after 5–7 days when synnemata of the hyaline *Chalara*-like conidial state (**Fig. 4A,B**) developed, clustered on the apothecium of a host fungus. Maximum conidium viability at seven days corresponded with rapid movements by immature nematodes and fungus-feeding and predaceous mites that apparently spread the conidia on the dung surface; the nematodes may be food for the mites. A week after dung deposition, perithecia developed in the vicinity of the synnemata, often growing though the synnemata. Ascospores matured rather slowly beginning within the ascus and continuing after ascus evanescence and ascospores were released passively to the perithecium tip. Ascospores were transported by predaceous mites 1–3 days after ascospore release (**Fig. 4F**). The second species, *P. spinuliformis*, developed more slowly compared to *Pyxidiophora* sp., with the hyphal conidial state of *P. spinuliformis* appearing 12–15 days after appearance of *Pyxidiophora* sp., and perithecia developing 3–4 days later on the same apothecia. Passive ascospore release to the perithecium tip corresponded in time with maturation of a different mite. Ascospore maturation coincided precisely with mite maturation judged by the number of attached ascospores compared.

The timing of life cycles of species on substrates other than dung is not well known. One example, *P. spinulorostrata*, has a longer incubation period than species growing on ephemeral substrates. With the help of John Webster, twigs were collected from the River Teign in Devon, England, the type locality of the species, and mailed to Baton Rouge, Louisiana, USA, where they were incubated in plastic containers at room temperature for observation (M. Blackwell, unpublished). About a month after collection, *Gabardnaudia*-like conidia developed, and about 7 days later, *P. spinulorostrata* perithecia appeared in association with *Heliscus lugdunensis*. No additional information, especially on potential vectors, is available. Conidia were not observed on the perithecial outgrowths as had been described (Webster and Hawksworth, 1986).

In addition to the need for a host, a second requirement for *Pyxidiophora* life cycle completion is escape from a depleted substrate. Insects, often beetles, are essential for targeted dispersal of the ascospore-derived morphs. Mites, however, may be of vital importance (Blackwell *et al.*, 1986a, 1986b, 1989). For example, *Pyxidiophora corallisetosa* and *P. kimbroughi* grow in the secondary phloem in bark beetle galleries (**Fig. 6B**). Bark beetles, however, mature in the outer bark of the tree requiring phoretic mites to transport the ascospores to the beetle vector. On substrates that can be observed, mites are more plentiful than beetles and they become very active, almost frantic in human terms, in moist chamber cultures coinciding with time of ascospore maturation (Blackwell *et al.*, 1986a; Blackwell and Malloch, 1989b).

Expanding Diversity

Easily discovered species of *Pyxidiophora* perithecial morphs have come from exposed substrates with abundant flying insects and phoretic mites. These include herbivore dung, fleshy fungi, beetle galleries in wood, decaying plant material, and beached seaweed known as wrack (Blackwell and Malloch, 1989a). Species occurring in bark beetle galleries, sometimes have been detected by observing the ascospore-derived conidial states on mites (Blackwell *et al.*, 1989). In fact, the wrack habitat was discovered by finding the ascospore-derived conidial state (*Thaxteriola*) attached to mites from wrack in the Natural History Museum, London. Unlike looking for a needle in a haystack, once a fertile habitat is discovered, *Pyxidiophora* species usually can be recollected. Some species have been discovered in BLAST searches of nucleotide databases. For example, the ITS sequence of *Pyxidiophora arvernensis* (CBS:657.82, GenBank accession number MH861535) was a close match with NCBI GenBank (accession number MF484620), "an uncultured Glomerales soil isolate." Is the sequence that of an arbuscular mycorrhizal (AM) fungus or is it more likely that of a *Pyxidiophora* using the AM fungus as a host? Several environmental ITS sequences from GenBank (see "Relevant Websites section"), the UNITE database (Abarenkov *et al.*, 2010), and Tedersoo *et al.* (2014) share $\geq$95% identity with sequences of known Pyxidiophorales (**Fig. 7**).

Laboulbeniopsis Clade

Laboulbeniopsis termitaria is an ectoparasite of termites, described from specimens collected in Grenada (Thaxter, 1920). The species was re-discovered fifty years later and is now known from Florida (Kimbrough and Gouger, 1970; Blackwell and Kimbrough, 1976b), Georgia (Blackwell, 1980a,b), Louisiana (Henk *et al.*, 2003), and Japan (Guswenrivo *et al.*, 2018). The minute thalli are under 150 μm in size and usually comprised of four superposed cells. The basal cell has a system of channels that secrete what is apparently a glue-like material attaching the thallus to the termite. This basal cell also contains a darkened pad, which seems diagnostic for *Laboulbeniopsis*. The elongated, and often brown in age distal-most cell produces spores at its base, which have been suggested to be ascospores. In fact, the attachment apparatus is almost identical with that of *Pyxidiophora* (see above) and *Coreomycetopsis* (see below). The presumptive asci evanesce and the presumptive ascospores are released through an apical ring at the thallus tip (Blackwell and Kimbrough, 1976b).

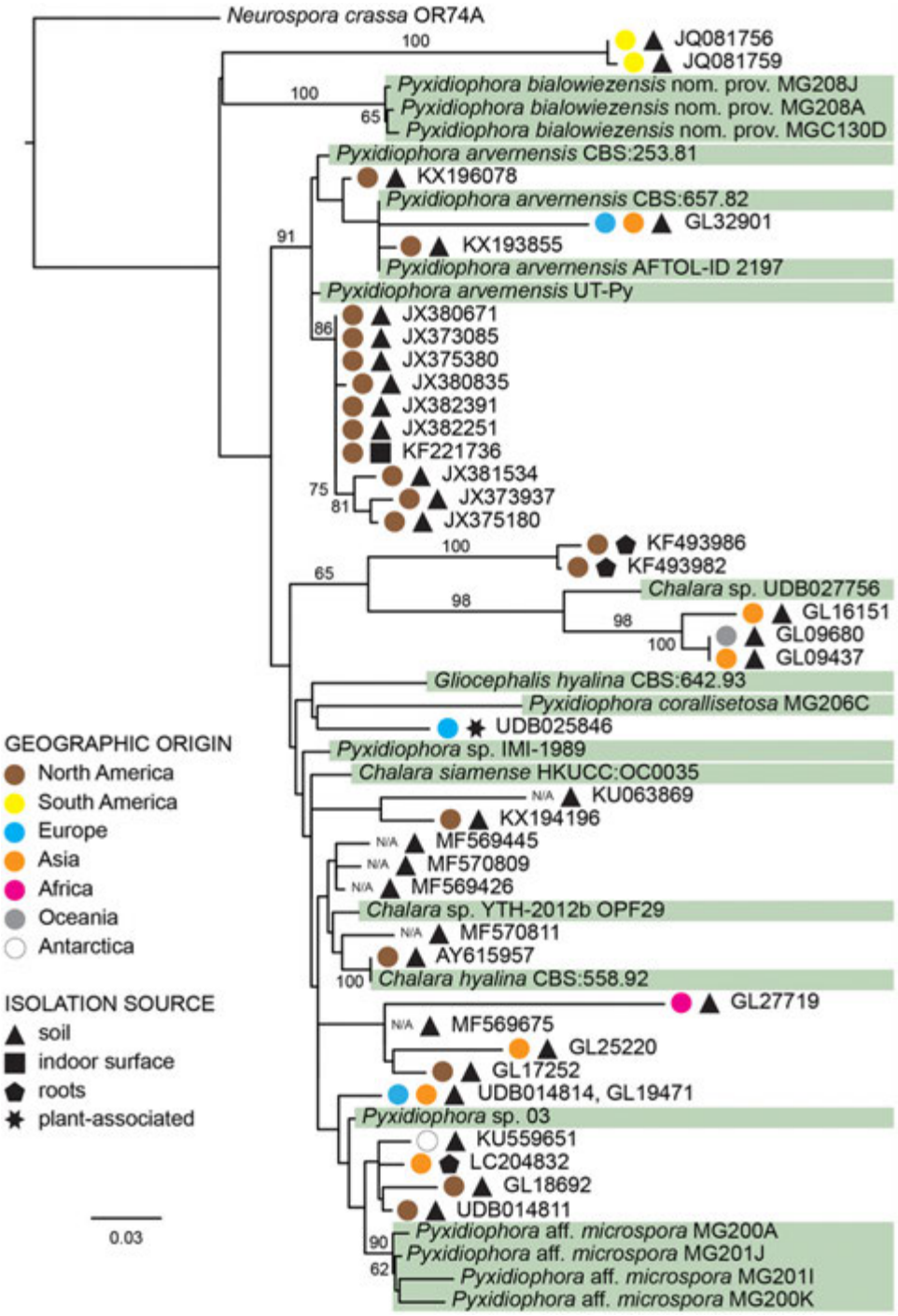

Fig. 7 Diversity of environmental ITS sequences of Pyxidiophorales. The topology is the result of maximum likelihood inference using RAxML (Stamatakis, 2014). Colored circles indicate geographic origin, black symbols indicate isolation source, sequences highlighted in green are known representatives of the order Pyxidiophorales.

An SSU rDNA phylogeny resulted in placed *Laboulbeniopsis* in the Laboulbeniomycetes with strong support (Henk *et al.*, 2003). Its placement was previously unknown even though it had been suggested to be associated with Laboulbeniomycetes based on morphology. More recently, Blackwell *et al.* (2020) found strong support for the sister relationship of *Laboulbeniopsis* and Pyxidiophorales in a two-locus rDNA phylogeny. The phylogeny revealed high support for five clades in the Laboulbeniomycetes: the orders Herpomycetales, Laboulbeniales, and Pyxidiophorales, and in addition the *Laboulbeniopsis* clade and the *Chantransiopsis* clade (**Fig. 1D**).

Coreomycetopsis oedipus, another minute fungus known only from termites, is placed in this clade based upon its morphological similarity to *Laboulbeniopsis*. The typical thallus of *C. oedipus* is under 150 μm in size and consists of fewer than fifteen superposed cells. Based on a report by Blackwell and Kimbrough (1976a), eight of the distal-most cells are obliterated by upward growth of thin filaments in the development of a "sporogonium." Some of the filaments developed as phialides and produced elongated phialospores. *Coreomycetopsis oedipus* is less often collected than *Laboulbeniopsis termitaria*, and although it may be less common, it is more difficult to discern against the pale termites because it has no dark pigments. It was described from Grenada (Thaxter, 1920) and since then recorded, in Florida (Blackwell and Kimbrough, 1976a), Georgia and Louisiana (M. Blackwell, unpublished), and Panama (D. Haelewaters, unpublished). Several mycologists suggested that *Coreomycetopsis* and *Laboulbeniopsis* may be different conditions or states of the same fungus, but morphological data are not supportive of this hypothesis (Blackwell and Kimbrough, 1976a,b). As already noted, at the ultrastructural level, the attachment region of *Laboulbeniopsis termitaria* and *C. oedipus* and *Pyxidiophora* are identical, though *Coreomycetopsis* lacks the darkened pad present in *Laboulbeniopsis*. Additionally, the terminal cells of the *Coreomycetopsis* thallus are thought to form a cavity where phialospores develop; a similar cavity in *Laboulbeniopsis* thalli is reported to produce ascospores in an evanescent ascus (Blackwell and Kimbrough, 1976b). The ultrastructural studies describing reproductive structures in these two termite-associated fungi need confirmation.

Chantransiopsis Clade

The *Chantransiopsis* clade comprises two conidial fungi known only as insect ectoparasites, *Chantransiopsis* and *Tetrameronycha* (**Fig. 1D**; Thaxter, 1914, 1920; Spegazzini, 1918; Rossi and Blackwell, 1990). Not much is known about these genera, other than their sparse filamentous growth occurs on insects. The other member of this clade is *Subbaromyces splendens*, a perithecial ascomycete with a distinctive collar-like structure at the base of the long perithecium neck, giving the perithecium the look of an injection syringe. *Subbaromyces splendens* was discovered on rocks in trickling sewage filter beds in New York, USA (Hesseltine, 1953). A second morphologically and ecologically similar species, *S. aquaticus*, was isolated from an open drain in Hyderabad, India (Manoharachary and Ramarao, 1974). Both species have limited growth in culture but produce conidia; production of perithecium and ascospores occurs only in mixed cultures. As with *Pyxidiophora*, these related fungi appear to have some dependence on other fungi, and perhaps are mycoparasites.

The genera *Chantransiopsis* and *Tetrameronycha* were for the first time included in the Laboulbeniomycetes by Goldmann and Weir (2018) in their SSU rDNA phylogenetic reconstruction of class members (**Fig. 1B**). Both genera were placed in a maximally supported clade in the phylogenetic reconstruction by Blackwell *et al.* (2020), presenting evidence for at least two clades with conidial states (*Chantransiopsis* clade, Pyxidiophorales) (**Fig. 1D**). Blackwell *et al.* (2020) generated sequences of *S. splendens* from a Hesseltine (1953) culture (CBS:357.53) and found high support for a placement among *Chantransiopsis* and *Tetrameronycha*. The inclusion of *Subbaromyces* in Laboulbeniomycetes was perhaps surprising, but the long-necked perithecia, evanescent asci, and accumulation of ascospores at the tip of perithecia are characteristic of other members of the class and suggests dispersal by arthropods.

Filling Knowledge Gaps

We close our discussion of the 170-year story of the Laboulbeniomycetes by pointing out missing chapters and pages. The basic outline for telling the complete story is a stable, well-resolved phylogeny that places these fungi within the rest of the ascomycetes; while we know that the Laboulbeniomycetes and Sordariomycetes form a monophyletic clade in the *Ascomycota Tree of Life*, the direct sister group of the Laboulbeniomycetes remains to be discovered. Progress in developing evolutionary hypotheses of the group will depend on (1) sampling new specimens, (2) studying the ecology, and (3) using multiple molecular markers and phylogenomic approaches.

Sampling new specimens of fungi associated as ectoparasites of arthropods but also free-living arthropod-dispersed fungi is crucial to obtain a complete picture of the diversity that is present in the class. Known host groups should continue to be investigated for the presence of Laboulbeniomycetes by both collecting in the field and screening museum collections. Existing literature will remain essential to point out hosts and localities in these endeavors. Priority should be given to those host species from which types have been described. Once infected specimens are found, sequences must be generated to define species limits and describe within-species phenotypic plasticity. In fact, not understanding the extent of morphological variation, obscures our conclusions concerning host shifting, specificity, and cryptic diversity.

Ecological aspects of Laboulbeniomycetes remain understudied. Climate effects on the distribution and survival of Laboulbeniomycetes and their hosts are poorly known. Focusing on ant- and bat fly-associated Laboulbeniales across Europe, Szentiványi *et al.* (2019) found that localities with low annual mean temperature and humidity, the likelihood of Laboulbeniales presence is higher. Welch *et al.* (2001) reported a prevalence of *Hesperomyces virescens* on *Adalia bipunctata* ladybirds as high as 54.7% in London, whereas prevalence had decreased to 0% at 25 km outside of the city. This extreme, short-range variation in prevalence was attributed to host phenology; the "urban heat island effect" shortens winters and may promote aphid growth – as do car pollution and urbanization. These factors increase host survival and enhance inter-generational contacts between ladybirds, providing ample opportunities for transmission of *H. virescens* (Welch *et al.*, 2001). Although these two examples seem to be

contradictory at first sight (lower versus higher temperature promoting development of Laboulbeniales), this illustrates that different Laboulbeniales may have different environmental preferences. More data are necessary from more parasite–host systems and larger geographical areas to pinpoint general patterns. A genomics approach has been used in a somewhat similar situation to look at fungal range extensions by detecting genome-wide differences in strains of the fungi involved that were associated with certain temperature and precipitation conditions across the range (Galagan *et al.*, 2005; Smith *et al.*, 2019).

Multiple molecular markers are already being applied to increase phylogenetic resolution and to discover additional members of the class among newly collected specimens. A significant milestone in Laboulbeniomycetes research, however, has been the acquisition of a 15-Mb draft genome sequence assembly of *Herpomyces periplanetae* (Haelewaters *et al.*, 2020b). An increasing number of reviews and research papers describe the use of genomics and proteomics applied to fungal mutualists (Biedermann and Vega, 2020) and pathogens of insects (Wang and Wang, 2017). Genome-scale data offer a short-cut to answering many questions, essentially turning any of the Laboulbeniomycetes into model fungi well suited for studies of symbiosis tracking interactions of fungi and their arthropod hosts.

Genomics will soon be used to predict reciprocal interactions of Laboulbeniomycetes and their associates. For example, basic questions about the *mode of nutrition*, can be addressed by an approach referred to as reverse ecology (Ellison *et al.*, 2011). In a study of endophytes of rubber trees, enzyme profiles were evidence of an unexpected insect-association rather than the expected plant-associated profile similar to that of the Xylariales (Gazis *et al.*, 2016). Correlations of variation in nutrition over parts of geographic ranges of broadly distributed species with genome changes (e.g., gene family expansions and enzyme family diversification) (Qian and Zhang, 2014), identification of genes unique to fungus–insect symbiosis (Wang *et al.*, 2018), and discovery of recurrent symbiont replacements by entomopathogenic fungi (Matsuura *et al.*, 2018), are the type of studies that will inform interactions among Laboulbeniomycetes and their associates. Are there other interactions, for example, signaling among Laboulbeniomycetes and loosely associated organisms (Becher *et al.*, 2018)? The tight associations suggest the possibility of horizontal gene transfer of siderophores found in microbe-packed insect guts (Tabima *et al.*, 2020).

Several species of Laboulbeniales and Herpomycetales occur on a *diverse variety of hosts* and have extremely *wide distribution ranges*, from subboreal to tropical areas. Molecular phylogenetic analysis and sequence-based species delimitation methods are necessary to evaluate whether these taxa are effectively ubiquitous, that is, capable of thriving on a wide range of hosts in many different microhabitats and climates, or whether they belong to separate cryptic or near-cryptic species. Examples are a number of taxa in *Laboulbenia*. One of the most widespread and most commonly sampled species is *L. flagellata*. Since its description (Peyritsch, 1873), it has been reported from more than 80 genera of Carabidae in many countries on all continents except Antarctica (Santamaría *et al.*, 1991; Majewski, 1994). Based on a limited LSU rDNA dataset, Haelewaters and De Kesel (2020) revealed three clades of *L. flagellata* sensu lato. It goes without saying that a lot more work will be needed to resolve the taxonomy of this and other species complexes. Genome comparisons have shown that variation occurs across taxa with broad distributions and have helped to identify genomic factors involved in adaptation in parts of the range (Galagan *et al.*, 2005; Smith *et al.*, 2019; Mei *et al.*, 2020).

Epilogue

Harkening back to the beginning of this chapter, the section "From Roland Thaxter to the Present: Synergy Among Mycologists, Entomologists, Parasitologists", and the mention of reviews at fifty-year intervals (Benjamin, 1971; Haelewaters *et al.*, 2021c), we anticipate a next comprehensive review – but likely many years before 2071. Major achievements in the taxonomy and systematics of Herpomycetales, Laboulbeniales, Pyxidiophorales, and related arthropod-associated fungi will be accomplished, but so much else can be discovered. We expect that significant progress based on molecular phylogenetic and phylogenomic studies, but also new collections, will speed the effort. But, who will there be write it? Perhaps a young student who is reading this chapter now.

Acknowledgments

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Pfliegler, Ana Sofia P.S. Reboleira, Walter Rossi, Sergi Santamaría, Isabelle I. Tavares, Rosa V. Villarreal Saucedo, and Alex Weir. And finally, where would we be without Cybertruffle, Index Fungorum, Index Herbariorum, museum collections, MycoBank, MyCoPortal, and the NCBI GenBank?

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