The origin of human pathogenicity and biological interactions in *Chaetothyriales*

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Abstract

Fungi in the order *Chaetothyriales* are renowned for their ability to cause human infections. Nevertheless, they are not regarded as primary pathogens, but rather as opportunists with a natural habitat in the environment. Extremotolerance is a major trend in the order, but quite different from black yeasts in *Capnodiales* which focus on endurance, an important additional parameter is advancing toxin management. In the ancestral ecology of rock colonization, the association with metabolite-producing lichens is significant. Ant-association, dealing with pheromones and repellents, is another mainstay in the order. The phylogenetically derived family, *Herpotrichiellaceae*, shows dual ecology in monoaromatic hydrocarbon assimilation and the ability to cause disease in humans and cold-blooded vertebrates. In this study, data on ecology, phylogeny, and genomics were collected and analyzed in order to support this hypothesis on the evolutionary route of the species of *Chaetothyriales*. Comparing the ribosomal tree with that of enzymes involved in toluene degradation, a significant expansion of cytochromes is observed and the toluene catabolism is found to be complete in some of the *Herpotrichiellaceae*. This might enhance human systemic infection. However, since most species have to be traumatically inoculated in order to cause disease, their invasive potential is categorized as opportunism. Only in chromoblastomycosis, true pathogenicity might be surmised. The criterion would be the possible escape of agents of vertebrate disease from the host, enabling dispersal of adapted genotypes to subsequent generations.

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Introduction

The order *Chaetothyriales* is particularly known through the derived family *Herpotrichiellaceae* containing the genus *Exophiala*. Most species of this genus initially propagate by budding, and are therefore known as 'black yeasts'. Nearly always the colonies become filamentous via inflation of the germinating cell leading to an intermediate 'torulose' mycelium (de Hoog et al. 1993), which is a morphological diagnostic feature for the genus. Related genera lack budding cells, and therefore the entire group of fungi has been referred to as 'black yeasts and relatives' (Seyedmousavi et al. 2014). These fungi are exceptional in the fungal Kingdom by colonizing hydrocarbon-polluted anthropogenic

habitats, and are also frequently encountered as opportunistic agents of disease in humans and cold-blooded vertebrates.

Chaetothyriales contains several less-known families. The order was proposed by Barr (1976) on the basis of sexual fruiting bodies collected on woody plant material. Those fungi produced small, setose, clypeate ascomata containing dark, multi-celled ascospores. The earliest classifications of the order were based on morphological characteristics, which allowed recognition of eight families (Barr 1987). *Chaetothyriales* are known in culture since only a few decades. In culture, no fruiting bodies are formed, and because of the poor morphological differentiation of the asexual states, DNA sequencing is required as a taxonomic approach, which yielded a system that deviated dramatically from the classical system and required rearrangements and repeated changes, and many new species and genera were introduced. Recent overviews accept five main families: *Chaetothyriaceae, Cyphellophoraceae, Epibryaceae, Herpotrichiellaceae*, and *Trichomeriaceae* (Chomnunti et al. 2012; Réblová et al. 2013; Gueidan et al. 2014; Quan et al. 2020), and some smaller groups. The families show differences in ecological preferences (Quan et al. 2020).

Genome analysis and genomic comparison of species have become a research hotspot in recent years. Since the first whole-genome sequence of a chaetothyrialean fungus, *Exophiala dermatitidis*, published by Chen et al. (2014), additional genomes have become available in increasing numbers each year. Up until now (24-08-2022), a total of 39 species, including 106 isolates were genome-sequenced and are available in the NCBI database. The early published genomes were mainly from some important human–pathogenic species, with a strong focus on the family *Herpotrichiellaceae*. Genomes from the ancestral families *Trichomeriaceae, Cyphellophoraceae, Chaetothyriaceae* and *Epibryaceae* have remained very scarce.

A rock-hard history

Basal lineages of *Chaetothyriales* are represented by black fungi living in various types of symbiosis with lichens, i.e. endolichenic fungi (Arnold et al. 2009) and lichen-associated fungi (Muggia and Grube 2018). Severe fluctuations of temperature and humidity are characteristic for sun-exposed rock surfaces that are the preferred habitat of lichens. These extreme conditions provide a cradle for species-rich communities of chaetothyrialean fungi. The lichen mycobiome changes with the ecological and growth parameters of the lichen host (Fernández-Mendoza et al. 2017; Banchi et al. 2018; Muggia and Grube 2018; U'Ren et al. 2019); the interactions with ancestral *Chaetothyriales* are manifold and still have to be unraveled.

Lichen-associated fungi cause diagnostic symptoms or formations on their lichen hosts, such as characteristic reproductive structures, hypertrophications, melanized and discolored patches, or local senescence of thallus (Lawrey and Diederich 2003; Diederich et al. 2018). However, more often they occur asymptomatically in the lichen thallus (Muggia and Grube 2018). The endolichenic fungi may have diverse degrees of specificity with their lichen photo- or mycobiont, or may reside in the intercellular matrix of the host (de los Rios et al. 2002). They grow relatively easily when isolated in culture, showing exclusively asexual propagation. Because of this, and the limited morphological differentiation shown from neighboring lineages in *Chaetothyriomycetidae*, their taxonomy rests on DNA sequence data and phylogeny. Chaetothyrialean lichen-associated fungi have been known for 30 years (Petrini et al. 1990), but their taxonomic diversity and phylogenetic position have been clarified only during the past decade (Hofstetter et al. 2007; Harutyunyan et al. 2008; Arnold et al. 2009; Muggia et al. 2015, 2016, 2017, 2019, 2021; Muggia and Grube 2018; Quan et al. 2020). Since the pioneering molecular studies of lichenized and non-lichenized rock-inhabiting fungi (RIF) in Chaetothyriomycetes by Ruibal et al. (2005, 2008) and the study of their evolutionary origins by Gueidan et al. (2007, 2008, 2009, 2011), the fungi were reclassified repeatedly under the pressure of a growing amount of sequence data. The ancestral *Chaetothyriales* in lichen-associated fungi and the RIF are of great interest because they are at the origin of the different lifestyles and habitats (Fig. 1), with *Pleostigmataceae* and ancestral relatives (Gueidan et al. 2008, 2011) as a cradle of diversification, possessing nascent properties that are further developed in derived families.

Four lineages of chaetothyrialean lichen-associated fungi have been characterized. (1) The family *Pleostigmataceae*, with three molecular sibling species in *Pleostigma*, was recently introduced (Muggia et al. 2021) as a sister group of *Verrucariales*. This lineage was previously recognized with two taxa (Stenroos et al. 2010) and confirmed in subsequent studies (Gueidan et al. 2014; Chen et al. 2015; Ekanayaka et al. 2019; Boonmee 2017). (2) A clade with an undescribed *Muellerella* species derived from the lichen *Rhizocarpon geographicum* is nested within *Verrucariales* (Muggia et al. 2019). (3) Another clade contains the lichenicolous species *Muellerella atricola, M. lichenicola* and *Lichenodiplis lecanorae* (Muggia et al. 2015, 2019), which together with *Epibryaceae* constitute a basal lineage of *Chaetothyriales*. (4) *Melanina gunde-cimermaniae* (Muggia et al. 2021) is a RIFs, associated with epilithic, crust-forming lichens in subalpine and Mediterranean habitats. The inferred phylogenetic position of the lichen-associated taxa supports their affiliation to bryosymbiotic taxa in the chaetothyrialean family *Epibryaceae*. The rock-inhabiting lifestyle is maintained
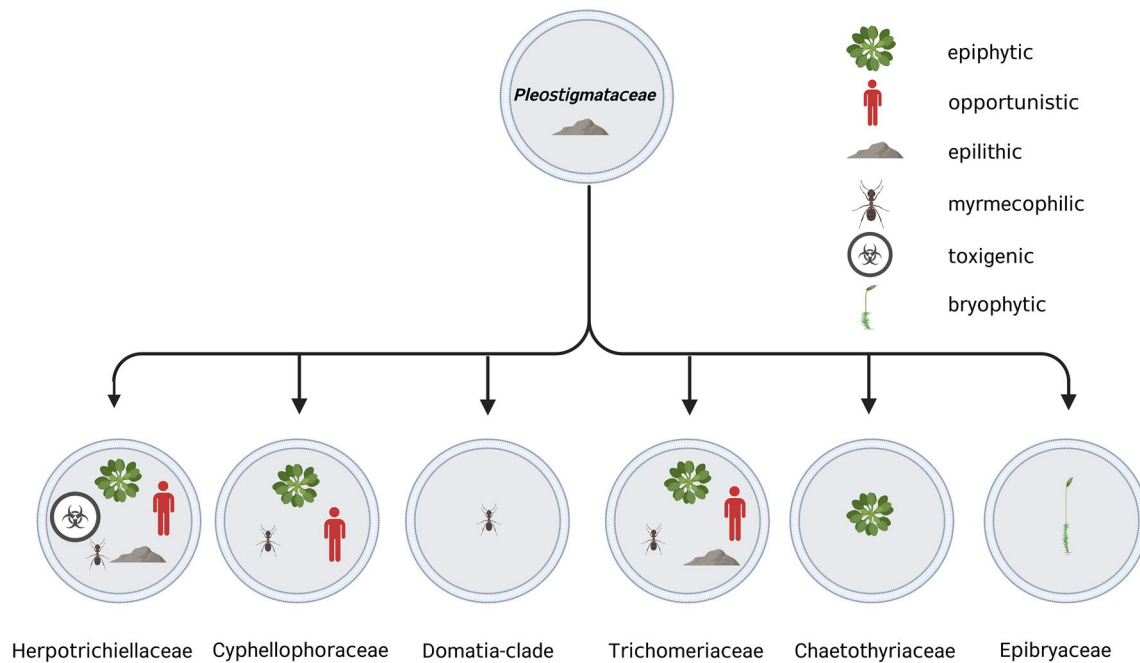
Fig. 1 Hypothetical ecophylogram of main ecologically relevant properties observed in families of *Chaetothyriales*. The lichen-associated lifestyle is taken as a cradle of diversification in derived families; most properties maintained or expanded in derived families are present in nascent form in *Pleostigmataceae*

across the derived corefamilies of *Chaetothyriales* (*Herpotrichiellaceae, Chaetothyriaceae, Cyphellophoraceae* and particularly in *Trichomeriaceae*) (Gueidan et al. 2008, 2011; Quan et al. 2020; Muggia et al. 2021).

The thallus of all chaetothyrialean members is consistently melanized. Melanin is an essential compound with numerous functions, enhancing survival under extreme conditions (Schnitzler et al. 1999; Moreno et al. 2017). Chen et al. (2014) found three independent melanin pathways in *E. dermatitidis*, chitin synthases, a diversity of transporters, and a full set of fungal light-sensing genes. Poyntner et al. (2018), using genomes of mutant and wild-type strains, showed that melanin significantly increases the invasiveness and virulence of *E. dermatitidis* in human hosts during the first day of skin infection. Pigmentation of cell walls may be protective against free radicals, enzymatic or microbial lysis, and extreme temperatures as well as contributing to resistance against host immune responses (Jacobson 2000; Zhang et al. 2013). However, the presence of melanin alone is insufficient to explain the pathogenicity of black yeasts, as this polymer is known to be present in many *Pezizomycotina*, of which only a fraction is able to infect humans or animals (Williamson et al. 2007). Remarkably, Song et al. (2017) noted that melanin is often lost when stress factors such as hydrogen peroxide are applied. Perhaps, in line with the above rock-inhabiting lifestyle, melanin is primarily effective against solar irradiation, and is just an accessory to other stress factors.

Teixeira et al. (2017) showed specific gene family expansions in 23 genomes of members of *Herpotrichiellaceae*: alcohol (*ADH*) and aldehyde dehydrogenases (*ALDHS*), membrane transport proteins, and a diverse ensemble of cytochrome P450 family members (*CYPs*), which all are considered as essential for survival under extreme conditions. Also a wide diversity of mechanisms associated with the nutrient acquisition was noted, matching with oligotrophy in line with the ancestral lifestyle on bare, sun-exposed rock (Quan et al. 2020). Derived groups of rock-colonizing species are found in the genus *Knufia* and relatives in the family *Trichomeriaceae* (Isola et al. 2016). All these fungi are extremotolerant, being able to thrive under conditions of high or low temperature, scarcity of nutrients, desiccation, and solar irradiation.

The hunger artists

Many members of *Chaetothyriales* seem to live on nothing. They have a competitive advantage in poor-nutrient habitats. In addition to rock association, a lifestyle colonizing plant leaves and other surfaces is widely distributed in *Chaetothyriales*, being found in *Herpotrichiellaceae, Cyphellophoraceae, Phaeosaccardinulaceae, Trichomeriaceae, Chaetothyriaceae*, and *Epibryaceae*. Such black fluffy overgrowth of inert substrates—rock, hard plant leaves—is often referred to as "sooty mold" (Chomnunti et al. 2014).

Unfortunately, strain isolation data in the literature are often unclear in that no attention is paid to whether the fungus on plants is epiphytic or invasive; detailed ecological studies are overdue. While the sooty molds are considered as epiphytes, the family *Herpotrichiellaceae* harbors some saprobic species involved in plant decay (Vicente et al. 2008, 2012; Nascimento et al. 2017), and recent data suggest that some may occur as endophytes (de Fátima Costa et al. 2022). Ancestral oligotrophy is recognized in prevalence in dishwashers (Raghupathi et al. 2018), bathing facilities (Matos et al. 2002) and household sinks (Nascimento et al. 2017) in the derived family *Herpotrichiellaceae*.

As an enigmatic group that is specialized in the habitat provided by *Bryophytes*, the family *Epibryaceae*, containing a single genus *Epibryon*, is phylogenetically affiliated to *Chaetothyriales*, although at some distance. The fungi biosynthesize secondary metabolites against competition and microbial damage (Xie and Lou 2009). Their asexual forms in culture consist of simple conidial chains and morphologically went unrecognized as *Cladophialophora* species, a genus of human opportunists in *Herpotrichiellaceae*. However, the *Epibryon*/"*Cladophialophora*" group forms a single clade in ancestral position in the LSU tree of the *Chaetothyriales* (Quan et al. 2020).

A toxic friendship

Lichens are known for their production and accumulation of toxic compounds (Dailey et al. 2008; Çolak et al. 2016). The ancestral lichen-associated fungi must therefore have developed the ability to tolerate or degrade these substances. The early history of *Chaetothyriales* thus has an element of survival of extreme physical conditions, as well as tolerance to polyphenols, shikimic acids, monoaromatic compounds, depsides, depsidones, diphenyl ethers, and dibenzofurans (Yousuf et al. 2014). This may have laid a foundation for diversification and adaptation to various types of more or less hostile habitats. Species of *Herpotrichiellaceae* are often isolated from awkward sources, such as hydrocarbon-polluted sites (Prenafeta-Boldú et al. 2001; Baron et al. 2021), gasoline and diesel car fuel tanks and fuel dispensers (Isola et al. 2013, 2021), and railway sleepers (Döğen et al. 2013)—habitats considered as hostile to fungal colonization. The toxins can even be applied for selective isolation of some species (Quan et al. 2019). Moreno et al. (2018) identified the genes linked to degradation of toluene by *Rhinocladiella mackenziei* by comparing the genome against the aromatic hydrocarbon degrading fungus *Cladophialophora immunda*. In the present paper, 13 genes were selected as a query to search from 45 genomes of *Chaetothyriales*. The copies of each gene per genome were calculated. This result was plotted on a phylogenomic

tree to analyze the distribution and evolution of the toluene pathway in *Chaetothyriales*. A significant difference in the copies of genes related to a toluene degradation pathway was observed between members of the derived family *Herpotrichiellaceae* (n = 12–23, av. 17.4) and those of the ancestral families lower down in the tree (n = 9–14, av. 11.4) (Fig. 2). The total number of these genes showed expansion, and toluene catabolism in the derived family was relatively complete, while in the lower families the pathway remained incomplete due to missing genes. Most of the investigated species in *Herpotrichiellaceae* were lacking only one or two genes, while the strains in the ancestral families missed four or five genes, indicating that their toluene pathway was far from mature. It can be surmised that the ability to decompose toxic substances has evolved over time.

Several *Exophiala* and *Cladophialophora* species common in the anthropogenic environment may rarely be found in nature. Possibly, relevant habitats have as yet not been explored. Examples are *E. dermatitidis*, for which a life cycle with fruit-eating tropical animals has been suggested (Sudhadham et al. 2008) and *Cladophialophora bantiana* that was recently encountered in sugarcane (de Fátima Costa et al. 2022). The virulence of opportunistic black yeasts may have been stimulated by adaptations to extreme environments. The combination of these features leads to polyextremotolerance (Gostinčar et al. 2018). Thermotolerance has also been considered as a virulence factor, but it does not always apply to *Chaetothyriales*, as many species cause infection in fish and amphibians (de Hoog et al. 2011).

Another toxic relationship concerns the uptake of heavy metals, i.e. any metallic chemical element with relatively high density and toxicity. Some are essential micronutrients for living organisms (e.g., Co, Cu, Cr, Mn and Zn), while others are nonessential (e.g., Cd, Pb, Ba, and Hg) (Tchounwou et al. 2012). Heavy metal ions exert their toxic effects in several ways upon exposure. They coordinate with biomolecules and may even replace essential metal ions exerting their toxic effect. Metals with redox properties also induce the formation of reactive oxygen species, leading to oxidative stress and cellular damage. Hence, oxidation states, ligands, solubility, and environmental conditions, are the factors determining the toxicity of heavy metals (Egorova and Ananikov 2017). In nature, the origin of heavy metals and metalloids is in weathering of mineral ores, but the molecules are emitted increasingly through mining and industry and have become important elements of environmental pollution (Briffa et al. 2020).

Some species of black yeasts, particularly in the genus *Exophiala* have a remarkable capacity to tolerate high levels of heavy metals in a solution that is toxic to most other organisms. *Exophiala sideris* has repeatedly been isolated from environments polluted with arsenic, tolerating As(V) with a half inhibitory concentration (IC50) (Seyedmousavi et al. 2011). *Exophiala mesophila* was also able to grow on Cr(VI)
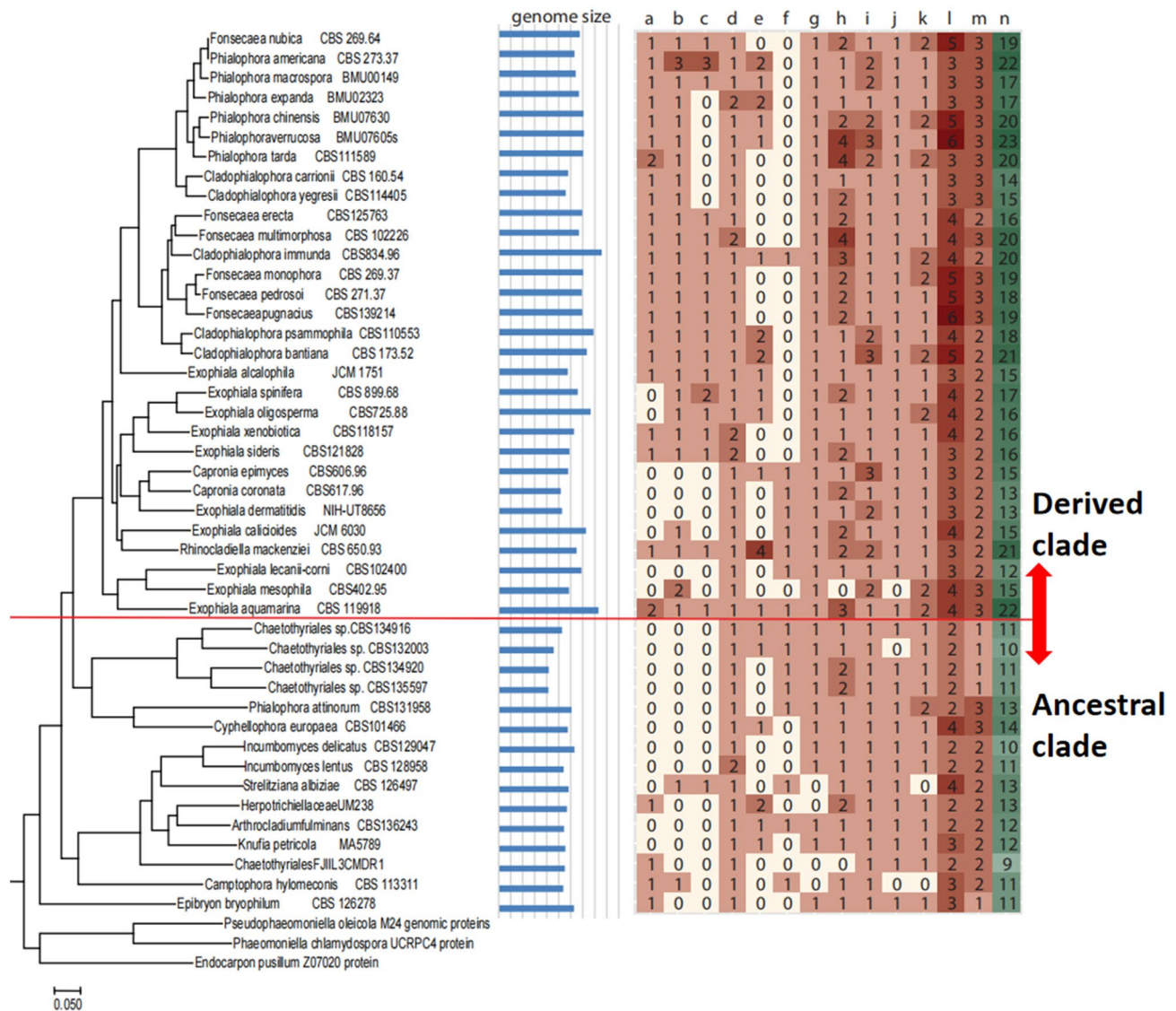
Fig. 2 Genome tree of 45 members of *Chaetothyriales*, combined with genes involved in toluene pathway shown at the right panel. A total of 13 genes were searched in 45 genome sequences. The lengths of the blue lines indicate the size of the genomes. These genes includ-ing five cytochrome P450 genes (a–e), dioxygenase (f), B-carboxy-muconolactone hydrolase (g), benzaldehyde dehydrogenase (h), benzyl alcohol dehydrogenase (i), muconate lactonizing enzyme (j), *p*-hydroxybenzoate hydroxylase (k), TR (l), TH (m)

at an IC50, which is well above the IC50 values reported for other microorganisms (Chen and Hao 1998). The aforementioned preference of some *Exophiala* species to grow on railway sleepers treated with tar-coal creosote, which contains high levels of heavy metals, matches with this behavior.

The fish-pathogen *Exophiala pisciphila* has also been isolated repeatedly from the roots of plants growing on soils that are polluted with heavy metals, and in vitro analyses have shown that this fungus tolerates concentrations of Pb(II), Cd(II) and Zn(II) at an IC50 of 0.8, 0.3 and 1.5 g/L, respectively (Zhang et al. 2008). Similarly, *Exophiala salmonis* was isolated as an endophyte from the roots of the

herbaceous plant *Astragalus adsurgens* (Chu et al. 2019); both species are closely related to *E. mesophila* above. The root-associated species *Exophiala radicis* and *Exophiala tremulae* (Maciá-Vicente et al. 2016) are members of the same clade. Endophytic colonization has been linked to the enhancement of plant tolerance to metal ions like Pb, Zn, and Cd (Cao et al. 2019). Melanin might confer metallotolerance to fungi and their host plants by acting as a quencher of reactive oxygen species generated by environmental stress, and by chelating and immobilizing toxic metals ions (Zhan et al. 2011). Additionally, transcriptomic data of *Exophiala pisciphila* exposure to Cd suggests that numerous

ATP-binding cassette transporter protein-encoding genes are involved in metal absorption and transport, contributing to the maintenance of metal homeostasis that confers adaptive advantages of *E. pisciphila* in metal-polluted environments (Cao et al. 2019). The latter feature could be engineered for the development of novel bioremediation techniques, and the prevention of the bioaccumulation of heavy metals in vegetable edible products. Possibly, the rhizosphere is overlooked as a source of *Chaetothyriales*, as also members of *Cladophialophora*, such as *C. chaetospira* and *C. inabaensis*, seem to form stable symbiotic associations with the roots of various plant species (Vanó et al. 2011; Santos et al. 2021). Even the neurotropic species *C. bantiana* was recovered from plant roots (de Fabiana Costa et al. 2022). Root colonization with *Exophiala* and relatives might play a vital role in the survival of plants under stress including the presence of toxic heavy metals, and other extreme conditions of temperatures, drought, salinity, and oligotrophy (Li et al. 2015a, b; Harsonowati et al. 2020; Xu et al. 2020). However, still little is known about the melanized fungi that colonize roots of healthy plants, and the numerous representatives from diverse orders are often dubbed as "dark septate endophytes" (DSE; Jumpponen 2001). A recent evolutionary assessment of the *Chaetothyriales* proposed that the DSE lifestyle, in analogy of endolichenic growth forms, might be an ancestral character of this order, which later diversified into multiple specialized forms (Suelgaray et al. 2023).

An itchy connection

The ability of *Chaetothyriales* to cope with extreme environmental conditions allowed them to use a rather special niche: ant nests. Strains are found (1) in cardboard-like "carton" structures used by ants to build their entire nest, to partition the space in natural cavities, to cover and protect their hemipteran trophobionts, or to construct runway tunnels and galleries for protection or for capturing large prey (Dejean et al. 2005; Schlick-Steiner et al. 2008; Mayer and Voglmayr 2009; Ruiz-Gonzalez et al. 2011; Voglmayr et al. 2011; Nepel et al. 2014; Mayer et al. 2017); (2) in nests of tropical leaf-cutter ants (Attili-Angelis et al. 2014; Duarte et al. 2014; Reis et al. 2015) and mounds of the boreal wood ants (Lindström et al. 2021); and (3) strains are frequently associated with ants living in symbiosis with plants in which the latter provide hollow structures called "domatia" for housing and very often also specialized food bodies for the nutrition of the ant colony (Nepel et al. 2014) (Fig. 3A–D). Symbioses with leaf-cutter ants and ants living with plants are restricted to the tropics around the globe (Voglmayr et al. 2011; Vasse et al. 2017) (Fig. 3E–H).

Although ant nests seem to be far from the usual habitats of *Chaetothyriales*, there are nonetheless crucial parallels.

Chaetothyriales that occur on outwardly exposed carton structures like galleries or nest walls, must be able to cope with high temperature, desiccation, extreme solar irradiation and probably nutrient scarcity as already described for the environment of rock colonizers above. The ecological function of "carton"-associated fungi might be to enhance the stability of nest walls and tunnel structures (Schlick-Steiner et al. 2008; Mayer and Voglmayr 2009; Ruiz-Gonzalez et al. 2011; Voglmayr et al. 2011; Mayer et al. 2017). This allows an improvement of the nest architecture as well as the development of peculiar defense and prey capture behaviour (Dejean et al. 2005; Mayer and Voglmayr 2009; Mayer et al. 2017).

Chaetothyrialean fungi within ant nests are exposed to a huge variety of chemical compounds produced by the ants to organize the colony, and protect the brood and adult nest members against pathogens (Hölldobler and Wilson 1990). Some ant pheromones such as pyrans, phenols, ketones, aldehydes and hydrocarbons functionally and structurally resemble those of the toxic lichen compounds described above (Morgan 2009; Vieira et al. 2010, 2012; Cerdá et al. 2014a, b). Mayer et al. (2021) analyzed the volatile organic compounds (VOCs) inside ant domatia in the tropical rainforest and hypothesized that ant-associated *Chaetothyriales* are important for cleaning the domatia air from volatiles that could be detrimental to the fragile larvae which are not protected by an exoskeleton. Interestingly, black yeasts have also been isolated from and used in biofilters treating air polluted with different VOCs, as reviewed recently by Prenafeta-Boldú et al. (2018). Blatrix et al. (2012) showed that chaetothyrialean fungi in domatia are sometimes used as food source for the larvae. Domatia fungi are actively grown throughout the lifetime of an ant colony in small delimited areas inside the domatia and are transported by the queen when founding a new colony (Defossez et al. 2009; Voglmayr et al. 2011; Mayer et al. 2018).

Phylogenetically, nearly all domatia fungi clustered in a single, yet poorly defined clade, while strains associated with carton are scattered in the families *Cyphellophoraceae, Herpotrichiellaceae*, and *Trichomeriaceae* (Voglmayr et al. 2011; Vasse et al. 2017). The deviating phylogeny and morphology of carton- and domatia-associated fungi suggests that *Chaetothyriales* has gone through different types of evolution with the ant-habitat as driver. The polyextremotolerance to harsh environmental conditions is probably rooted in rock-inhabiting *Pleostigmataceae* and allies and has enabled slow-growing *Chaetothyriales* with low competitive ability to successfully use ant-shaped niches where they are superior to many other fungi.

However, at the genome level, we have not yet found a strong adaptation to the ant environment that crosses family boundaries. This is most likely due to the small number of genomes of ant-associated fungi that have been analyzed so

Fig. 3 Carton galleries of *Azteca brevis* along the stem and branches (**A**, **B**) with little holes. The dense net of hyphae is stabilizing the gallery structure (**C**). Chaetothyrialean fungi are invading a newly built gallery part made of scratched plant particles (**D**). Patches of domatia fungi inside the stem of *Cecropia obtusifolia* inhabited by *Azteca alfari* (**E**, **F**) and inside branches of *Triplaris melaenodendron* inhabited by *Pseudomyrmex* sp. (**G**, **H**). Scale bars: 200 μm (**C**), 100 μm (**D**). Photo credit: M. Nepel (**D**), R. Blatrix (**G**, **H**)

far. Genomes of only two carton-fungi are available to date: *Incumbomyces delicatus* and *I. lentus* (Quan et al. 2021), belonging to *Trichomeriaceae*. These species differ considerably from the genomes of four domatia fungi, clustering in the "domatia clade". Domatia-colonizing black fungi have remarkably small genomes compared to other *Chaetothyriales*, with a large number of gene family contractions, especially in carbohydrate-active enzymes (CAZymes) up to a complete loss of some polysaccharide lyase families

(Moreno et al. 2019). Genomes of carton fungi had a much higher total number of CAZymes (Quan et al. 2022). Domatia fungi show a remarkable increase of gene clusters related to secondary metabolism (Moreno et al. 2019) like modular polyketide synthases (type-I PKS) and nonribosomal peptide synthetases (NRPSs). Type-I PKS and NRPSs are known to be involved in the synthesis of some of the most important antibiotics and antiparasitics (Fischbach and Walsh 2006; Süssmuth and Mainz 2017) and their increase suggests a

certain function in the mutualistic relationship with the ant-plant system.

Remarkably, the *Incumbomyces* carton fungi were more similar to the human opportunistic species *Arthrocladium fulminans* (member of the same family *Trichomeriaceae*) in genome size, gene number, and GC content, rather than to the domatia species (Moreno et al. 2019, Moreno et al. 2020; Quan et al. 2022). The difference might be caused by the site of growth: on the surface of nest walls or galleries, or inside domatia. The carton fungi investigated are surface colonizers of abiotic environments and thus would be ecologically somewhat similar to remaining members of *Trichomeriaceae*, a family mainly comprising surface-colonizing fungi (Chomnunti et al. 2012; Isola et al. 2016). Both species of *Incumbomyces* lack conidia. With age, the hyphae tend to transform and liberate as a meristematic, isodiametric type of cells, as is commonly known with RIF on exposed surfaces. Sexual states are unknown, but *Incumbomyces lentus* has both *MAT1-1* and *MAT1-2* idiomorphs, a condition thus far only proven in sexual *Capronia* species (Teixeira et al. 2017). The species may thus have a homothallic sexual cycle in nature.

Given the biodiversity, wide distribution, and different nesting activities of ant species, as well as specific functions in ant–plant associations, a rich diversity of fungi associated with these arthropods has already been found (Mayer and Voglmayr 2009; Voglmayr et al. 2011; Nepel et al. 2014; Quan et al. 2021) and is also expected in further investigations.

The dirty pictures

Infections by black yeasts and relatives are renowned because they may lead to severe mutilation and death. The neurotropic species *C. bantiana* has been placed in the top-10 of most feared fungi (Hyde et al. 2018). Several cases of chronic, recalcitrant infection, severe mutilation and slow death of supposedly healthy individuals have been reported in somewhat older literature (Hiruma et al. 1993; Chang et al. 2009; Alabaz et al. 2009). The recent discovery of rare inherited immune disorders in humans, particularly mutations in the caspase recruitment protein domain 9 (*CARD9*) has shed new light on these clinical syndromes (Glocker et al. 2009). It mediates signals from Dectin pattern recognition receptors (PRRs) that activate pro-inflammatory cytokines, and via NF-κB activation interleukin Th-17 is released (Fig. 4). When cytokines are deficient due to a patient's *CARD9* mutation, the host should be susceptible to numerous fungi. However, these patients are usually infected by only a single fungal species, which mostly belongs to *Candida*, dermatophytes, or *Chaetothyriales* (Vaezi et al. 2018; Song et al. 2020). Remarkably, the most common

opportunist genus *Aspergillus* is nearly absent in *CARD9* patients. This suggests that the dermatophytes, *Candida* and black fungi share something that other fungi such as *Aspergillus* don't have. Thus far, *E. dermatitidis, E. spinifera*, *Veronaea botryosa* and members of the *Phialophora verrucosa* complex have been reported in association with *CARD9* deficiency (Song et al. 2020), but probably all classical cases of severe, chronic, highly mutilation and often fatal infections by *Chaetothyriales* fungi are explained by this or a related (GATA2; Egenlauf et al. 2019) inherited immune disorder. The prevalence of *Chaetothyriales* is even more remarkable, as most of these fungi are uncommonly isolated from humans and the environment, which is in contrast to *Candida* and dermatophytes which are commonly present on asymptomatic hosts. Genomic work to understand these special abilities of *Chaetothyriales* is still in its infancy and will be a major task in future research.

The most common disease inflicted by members of *Chaetothyriales* is chromoblastomycosis (CBM). This is a (sub) cutaneous infection where the agent in tissue converts to isodiametrically expanding "muriform cells" (Queiroz-Telles et al. 2017a, b). Phagocytosis, creating low pH inside the macrophage, favors this cell type which is phagocytosis-resistant (Karuppayil and Szaniszlo 1997). Cellular host response is with acanthosis, mostly leading to hyperkeratosis or nodular formations elevated from the skin surface (Fig. 5). The disease is chronic and difficult to cure, and still prevails in rural tropical regions of Brazil (Wagner Santos et al. 2020), Venezuela (Pérez-Blanco et al. 2006), Madagascar (Rasamoelina et al. 2020) and South China (Lu et al. 2013). The disease has been recognized as a "neglected tropical disease (NTDS)" by the World Health Organization (WHO 2017; Hay et al. 2019). Clinically, there is some resemblance with *CARD9*-related infections in its chronic course, cutaneous dissemination, and nodular eruptions. Some of the patients indeed proved to have a non-synonymous mutation in *CARD9* CDS (Sobianski et al. in prep.). Other patients with *CARD9* defects showed necrotic patches with hyphae in tissue, consistent with phaeohyphomycosis (Huang et al. 2019). *CARD9* thus seems to aggravate existing infection types.

The *Chaetothyriales* contain many agents of human infection, ranging from severe to nearly asymptomatic. Species are distributed over 11 genera in three derived families: *Herpotrichiellaceae* (*Cladophialophora, Exophiala, Fonsecaea, Phialophora, Rhinocladiella, Veronaea*), *Trichomeriaceae* (*Arthrocladium, Knufia, Bradymyces*) and *Cyphellophoraceae* (*Anthopsis, Cyphellophora*) (de Hoog et al. 2020). In addition to infecting humans, infections in cold blooded water-borne animals are common, such as fish, frogs, toads, turtles and crabs (Boeger et al. 2005; de Hoog et al. 2011, 2022); remarkably, infections in warm-blooded animals other than humans are nearly lacking.
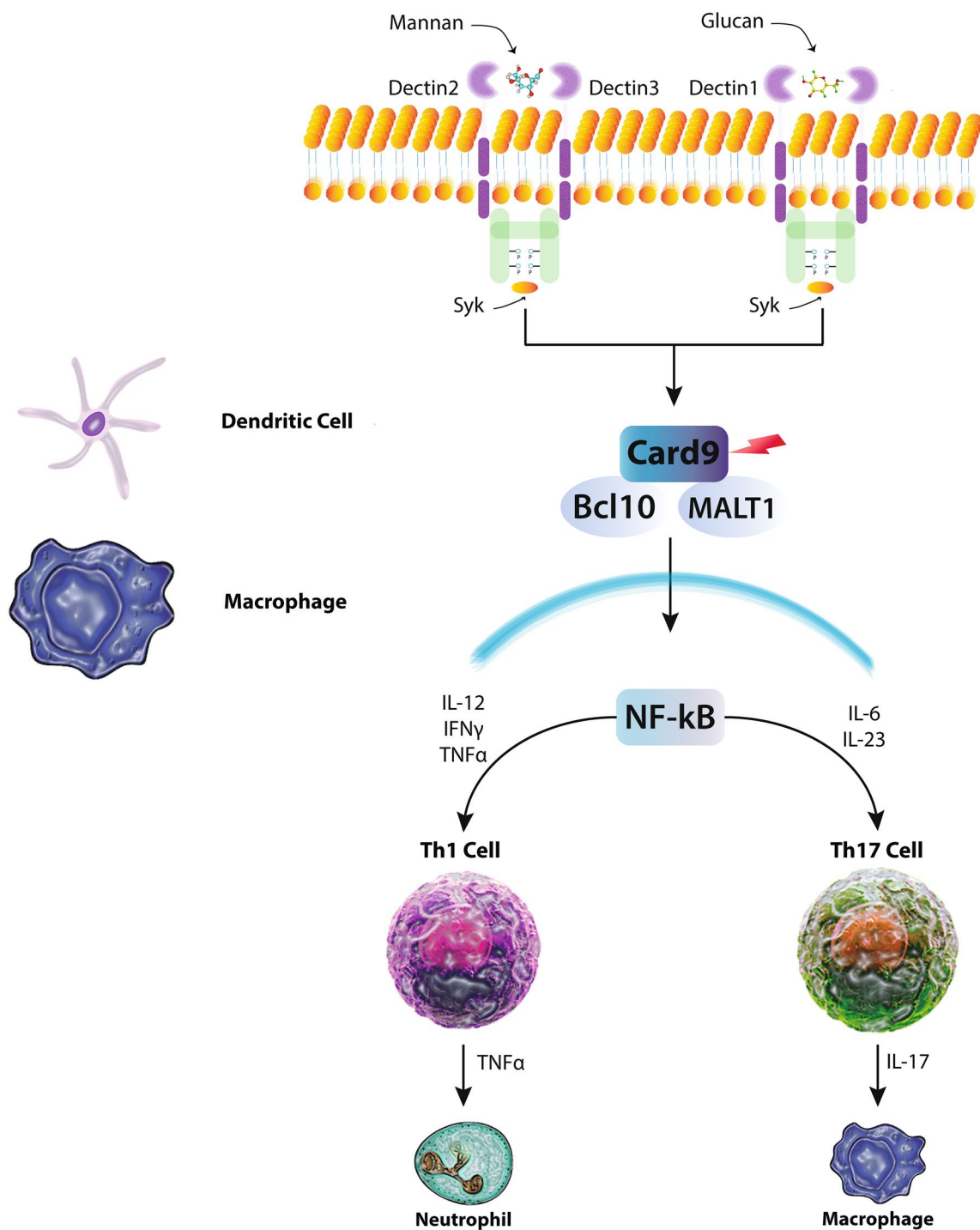
Fig. 4 Diagram of signaling pathway via Dectin and CARD9 triggering neutrophil and macrophage

CBM is almost entirely restricted to *Herpotrichiellaceae*. Rare exception were published in *Gloniopsis percutanea* [*Hysteriales*, published by Chowdhary et al. 2008 as *Rhytidhysteron*] and *Phoma insulana* (*Pleosporales*, Hernández-Hernández et al. 2018). Primary cerebral phaeohyphomycosis in otherwise healthy patients is also very rare

outside *Herpotrichiellaceae*. The ability to infect vertebrate hosts culminates in this family. According to data available in MEDLINE, most of the cases reported during the last 10 years were caused by species of *Fonsecaea* and *Cladophialophora*, with the prevalent agents being *F. pedrosoi* (353 reported cases), *F. monophora* (113 reported cases), *F.*
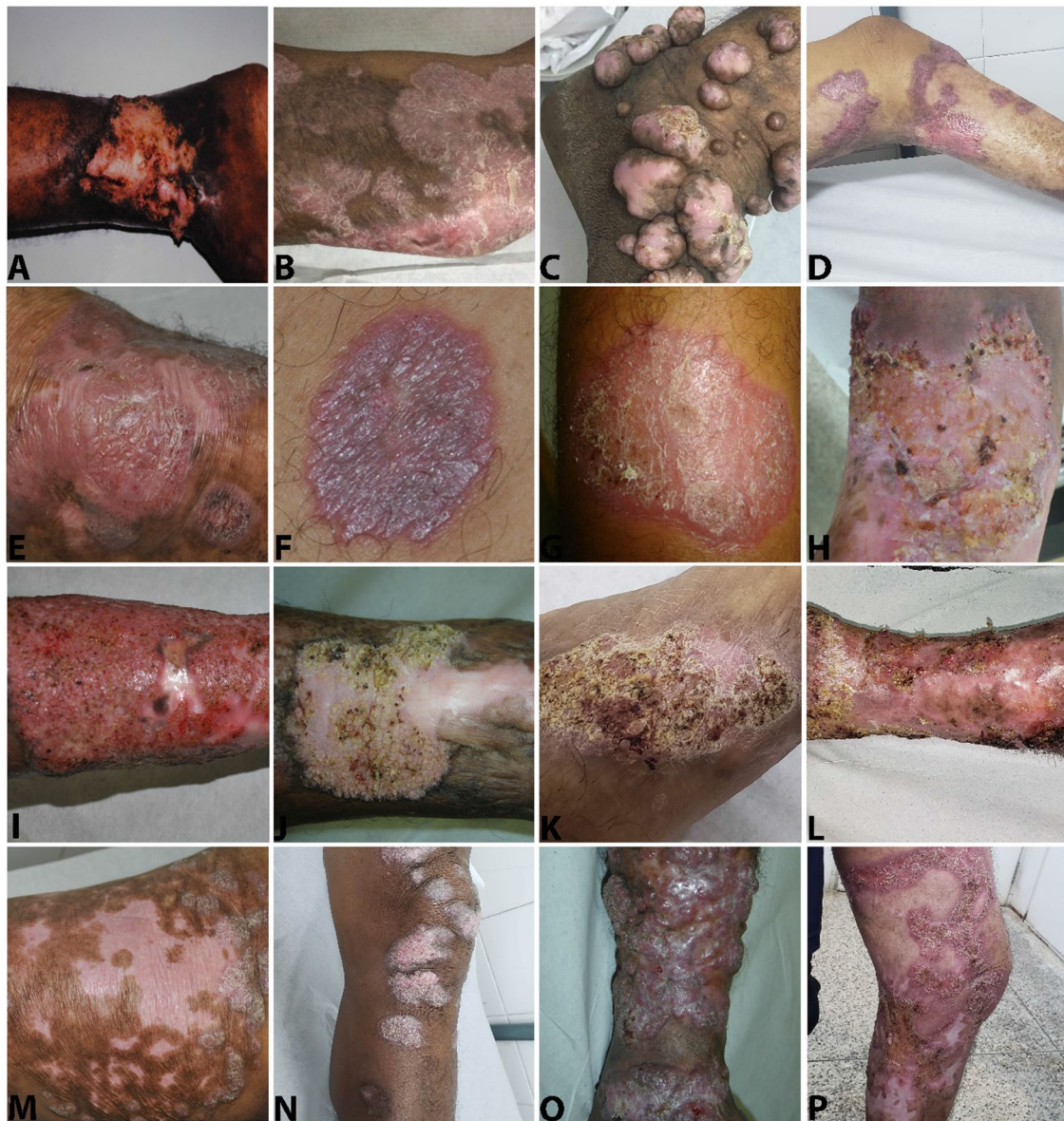
Fig. 5 Clinical manifestation of chromoblastomycosis caused by *Fonsecaea pedrosoi*. (CMRP 1292) **A** scar lesions, (CMRP 2306) **B** scar and plaque lesion, (CMRP 1278) **C** tumor and nodular lesion, (CMRP 1295) **D** lesions in recurrent infiltrative plates on scar edge, (CMRP 2307) **E**, (CMRP 1281) **F**, (CMRP 1303) **G** and (CMRP 1298) **H** plaque lesions, (CMRP 1299) **I** and (CMRP 1309) **J** veg- etating lesions, (CMRP 2308) **K** verrucous lesions, (CMRP 1280) **L** polymorphic lesion with nodules and verrucous edges, (CMRP 1316) **M** papular and vegetating nodular polymorphic lesion, (CMRP 1298) **N** papular, nodular, and infiltrative polymorphic lesion, (CMRP 1271) **O** tumor, nodular and vegetating polymorphic lesion, and (CMRP 1281) **P** polymorphic plaque and vegetative lesion

nubica (75 reported cases), and *Cladophialophora carrionii* (53 reported cases) (Hoog et al. 2007; Queiroz-Telles and de Santos 2013; Krzyściak et al. 2014). Recently published new agents of the disease are also members of *Chaetothyriales*, among which are *Fonsecaea pugnacius* (de Azevedo et al. 2015) and *Cyphellophora ludoviensis* (Gomes et al. 2016).

As described above, the genera that are frequent as opportunists also have a predilection for the hydro-carbon-polluted anthropogenic environment. Ongoing specialization with hydrocarbons is exemplified by the ability of several species to biodegrade and even assimilate several toxic monoaromatic structures as the sole source of carbon and energy (Blasi et al. 2016). Prenafeta-Boldú et al. (2006) introduced the term "dual ecology" for this ability. Their frequent infection of human hosts suggests that they may use similar substances in the body as in the polluted environment. A possible connection is found in the chemical structure of several neurotransmitters such

as adrenalin and noradrenalin, which are catecholamines and share L-tyrosine and dopamine with the melanin pathway. This relationship may be useful when the fungus is inside the vertebrate body, but as black yeasts have no specific means to enter this body, nor to escape from it after death of the host, they are unlikely to adapt to pathogenicity. Despite the sometimes severe clinical pictures of *Chaetothyriales*, we hypothesize that the members are opportunists having an environmental niche, rather than primary pathogens. The main reasons to classify them as opportunists can be summarized as follows: (1) almost all clinical fungi are found in the environment and lack host-specific adaptations to conditions in the host, as we know from environmental pathogens; (2) the route of infection mostly seems to be traumatic from an environmental focus (although there are exceptions to this rule such as the enigmatic neurotrope *C. bantiana*); (3) after infection, the hosts are not contagious and the fungus does not seem to have mechanisms to return to the environment as known, e.g., for dermatophytes. Lacking transfer and survival after

infection, the use of an animal host thus decreases rather than increases the species' evolutionary fitness (Fig. 6).

A pathogenic specialty

Perhaps there is an exception to the above conclusion. If *Chaetothyriales* are just opportunists, how can they be so frequent, with such species diversity, in a circumscribed disease as CBM? The muriform cell (MC) in host tissue makes this unique in the fungal Kingdom, but it is nevertheless polyphyletic within the *Herpotrichiellaceae* (Queiroz-Telles et al. 2017a, b). Extremotolerance with survival of phagocytosis, toxin management, efficient nutrient acquisition, thermotolerance, the catecholamine connection, the MC conversion and possibly microaerophily accompanying endophytic growth (de Fátima Costa et al. 2022) all enhance survival in tissue. Carpouron et al. (2022) identified transmission—which signifies advantageous use of the host by the fungus—as a decisive criterion of pathogenicity, since this enables
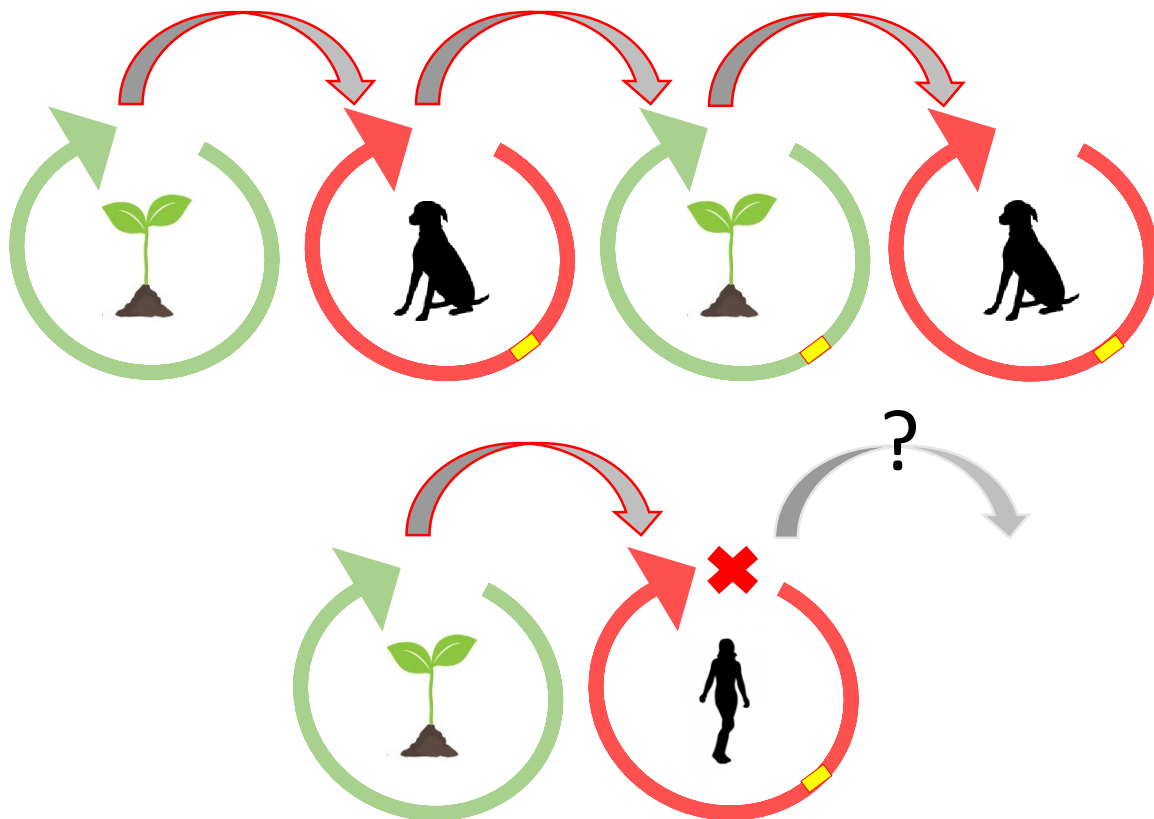

Fig. 6 Diagram of life cycles of an environmental pathogen *versus* an opportunist. **A** Environmental pathogens have a preferred habitat in nature to complete their assimilative thallus with sexual cycle, using nutrients produced by a host animal. The animal host is used for optimal distribution within its endemic area. Fungal mutations acquired within the host can be transmitted to the next generation allowing adaptation. **B** Opportunists have non-animal related habitats

and are exceptionally introduced into an animal host. They usually die with the host's end of life span, or are shed in the environment with an extremely small chance to reach a next animal host. Mutations acquired within the host are not transmitted to the next generation, and no adaptation takes place. (?) The high frequency of human chromoblastomycosis might balance this chance of reinfection, in which case the opportunist may evolve into a pathogen

transfer of adaptations acquired inside the host to the next generation (Fig. 6). Without transmission, it is difficult to see how environmental fungi can gradually become more pathogenic as assumed in a hypothesis on stepwise increase of pathogenicity in the course of evolution (Casadevall and Pirofski 2003; Casadevall 2006). An essential research question would therefore be, whether agents of common diseases such as CBM are able to escape from the living host by shed fungus-loaded skin flakes or cutaneous material, and to return to the environment, similar to, e.g., dermatophytes (Hashimoto 1991). In that case, we would observe a transition from an opportunist (with no evolution towards pathogenicity) into an environmental pathogen.

Causative agents of CBM are mainly found in *Fonsecaea, Cladophialophora* and *Rhinocladiella*, with *F. pedrosoi, F. monophora,* and *C. carrionii* as major agents. While *Fonsecaea* species are restricted to tropical rainforests, *Cladophialophora* is found under semi-arid conditions (Xi et al. 2009). Vicente et al. (2017) tested the virulence of *Fonsecaea* sibling species in a *Galleria mellonella* infection model, and observed that larvae infected by environmental *F. erecta* had lower survival rates than those infected by clinical strains of *F. pedrosoi*, but differences were very small. Virulence tests using *Tenebrio molitor* confirmed this conclusion (Fornari et al. 2018). Detection of antigenic components using BALB/c mice infected with *Fonsecaea pedrosoi, F. erecta* and *F. monophora* (Vicente et al. 2017) revealed that the plant-associated species *F. erecta* was more immunogenic than the clinical species, as evidenced by antigens in the serum of infected mice. Fornari et al. (2018), using plant infection models, suggested that all *Fonsecaea* species were saprobic, considering the fact that all agents have to be traumatically inoculated to cause disease, and thus primary animal pathogenicity is unlikely to be present. Liu et al. (2019) underlined the significance of melanin in the pathogenicity of *F. monophora* in a *G. mellonella* model, the albino mutant being found highly virulent to the heterologous larva host. Melanin could activate an immune response and promote the formation of inflammatory nodules in the larvae. Stappers et al. (2018) had identified a melanin-sensing C-type lectin receptor (MelLec) which has a key role in protective antifungal immunity. Given the fact that MelLec homologues exist in invertebrate models such as *G. mellonella* (Casadevall 2018), their MelLec homologue-mediated activated immune system may account for the lower virulence of the melanized strains to *G. mellonella* (Liu et al. 2019).

The host strikes back

The immune response of patients with severe forms of CBM showed low levels of IFN-γ and T-cell proliferation, while the opposite was observed in patients with mild forms

(Gimenes et al. 2005). The Th1 anergy was reverted when the patients' T-cells were incubated with homologous dendritic cells (DC) pre-incubated with conidia, showing the capacity of DC to increase the inflammatory modulation in this disease (Sousa et al. 2009). The T-cell response is important for the disease outcome, as demonstrated in the nude mice model which mimics the severe form of the disease (Ahrens et al. 1989).

Patients with CBM usually originate from rural areas. They attend health care institutions only after a long time of infection, when the adaptive immune response is established. The importance of patients' T-cells corroborates with outcomes of the murine model. In mice, CD4+ cells are more important than CD8+ cells (Teixeira de Sousa et al. 2006), and the CD4+ cytokine profile also modulates the progression of the lesions. Siqueira et al. (2017) demonstrated that the presence of Th17 in the lesion is associated with lesion progression and higher fungal burden, while Th1, with IFN-γ production, is associated with a cure. IL17 (Silva et al. 2014) and beta-defensin 2 (Leeyaphan et al. 2016) were described in skin of patients with CBM which showed a Th17 response at the lesion.

Fonsecaea species exhibit three morphotypes, which interact differently with host tissue. Conidia and hyphae are the infective forms, while the MCs are the pathogenic form. Each morphotype induces a different type of inflammatory response. Initially, the infective forms, especially the hyphae, elicit low inflammatory reactions. However, the pathogenic form is responsible for inflammation and persistence in tissue, with the production of pro-inflammatory cytokines, gene expression up-regulating of fungal recognition, inflammation, and phagocytosis. The inflammation is sustained by macrophage recognition since the MCs were phagocytosed only by PRRs Dectin-1 and FCgR (Siqueira et al. 2017). This difference in inflammatory reaction could improve the latency status of conidia in macrophages, since this morphotype did not require any PRR to be internalized by macrophages, generating low cellular activation.

At onset of infection, a mechanism of fungal communication through extracellular vesicles (EVs) is required. The EVs obtained from cultured conidia induced significant levels of inflammatory cytokines, while EVs from media with MC showed an anti-inflammatory profile (Las-Casas et al. 2022). These results corroborate that conidia do not elicit an inflammatory response in situ, but may have an impact on the type of tissue reaction. At the same time, the MCs maintain the inflammation in situ, and it is not necessary to increase the inflammation through EVs.

Development of chronic CBM infection is also associated with innate recognition characteristics. The absence of fungal recognition by Toll-like receptors (TLR) is due to inappropriate innate immune response activation, especially TLR-2 and TLR-2 (Sousa et al. 2014; Breda et al. 2020).

Successful treatment with imiquimod for CBM signifies the importance of TLR in activating the host's protective response (de Sousa et al. 2014). Fungal recognition by exclusively C-type lectin receptors (CLR) was insufficient for a protective response, especially when the Dectin-1 receptor which is essential for immunity to fungi remains inactive. CLR Mincle induces a Th2 rather than Th1 polarization, since IL-12A transcription is blocked through nuclear IRF-1 inactivity (Wevers et al. 2014). However, a synchronized recognition through TLR/CLR Mincle/Syk/CARD9 pathway can adequately activate the host's immune cells (Sousa et al. 2014). In addition to the role of Dectin-1 and Mincle, the Dectin-2/FcRγ/Card9 signaling pathway promotes fungus-specific Th17 differentiation (Robinson et al. 2009; Wüthrich et al. 2015), cytokine release associated with lesion progression, and fungal growth in the tissue (Siqueira et al. 2020). Consequently, CLR Dectin-1, Dectin-2, and Mincle activate different pathways which do not stimulate a protective adaptive immune response in CBM.

Genomic comparisons of environmental and clinical species showed expansion of protein domains such as glyoxalases and peptidases, which is in line with human infective abilities, while genes for nitrogen assimilation and degradation of phenolic compounds were enriched in environmental species (Vicente et al. 2017). Tolerance of extreme conditions is shared by many members of *Herpotrichiellaceae*, enhancing opportunistic tendencies in *Fonsecaea* and *Rhinocladiella* (Fornari et al. 2018). This was even more pronounced in endemic species of arid climates (Moreno et al. 2018). The virulence of *C. carrionii*, the CBM agent in semi-deserts, and its environmental counterpart *C. yegresii* was investigated in a *Galleria mellonella* infection model. Infective abilities were strain-dependent, and remarkably high virulence was also observed in one of the environmental species. Survival of larvae also varied with infection dose, growth speed and fungal melanization.

Kill'm all!

Virulence of chaetothyrialean black yeasts may be unpredictable, and a large variation in degrees of antifungal susceptibility and treatability is observed. In general, infections are known to be recalcitrant. The disseminated infections that we now know to be *CARD9*-related are particularly infamous; practically none of classical treatment options is effective. Only stem cell transplantation, as successfully applied in dermatophytes (Queiroz-Telles et al. 2019) seems a last resort. Since members of *Chaetothyriales* are involved in a wide diversity of infections, ranging from superficial infections to CBM and phaeohyphomycosis with or without central nervous system involvement, appropriate clinical management differs with the type of disease.

Also the in vitro antifungal susceptibility profile of the etiologic agent needs to be considered. Of the prevalent mycoses, CBM is chronic and very difficult to eradicate, sometimes resulting in amputation when the affected body area is too extensive, and also cases of phaeohyphomycosis may result in disfiguring and show relapse. Remarkably, however, the etiologic species may be in vitro susceptible to the used antifungal. For example, *C. carrionii* has low MIC values against ITZ, VCZ, TBF, 5FC, PCZ, ISA, and VCZ (Vitale et al. 2009; Deng et al. 2013), and for *Fonsecaea* species low MIC values for ITZ, VCZ, PCZ, ISA have been obtained (Label et al. 2018; Najafzadeh et al. 2010). Species of *Exophiala*, the prevalent genus involved in various types of phaeohyphomycosis, showed low in vitro values for VCZ, TBF, ITZ, PCZ, ISA (Vitale et al. 2003; Najafzadeh et al. 2021; Badali et al. 2011).

A meta-analysis recently of a large body of data collected in the Atlas of Clinical Fungi (de Hoog et al. 2020), the *Chaetothyriales* were demonstrated to be among the orders in the fungal Kingdom with low antifungal resistance and high intra-ordinal variation (Niu et al. 2022). MIC90 and GM data demonstrated pronounced azole susceptibility, with very few deviating strains. Percentages of resistance were generally below 10%, while melanized environmental opportunists in *Capnodiales, Dothideales, Pleosporales, Sordariales* and *Venturiales* all showed 50% or more counts in the resistant range to ITZ, PCZ and VCZ (Niu et al. 2022). Thus, despite low MIC values for the most commonly used drugs in clinical settings, infections by members of *Chaetothyriales* can be a challenge to cure. The exact mode of action of the drug in relation to the host's immunity will remain an important research question to better understand why diseases relapse, acquiring resistance under therapy.

Too early for name changes

Three types of taxonomic concepts have been used in *Chaetothyriales*. (1) Prior to the year 2000, description of species was limited to the phenotype. In environmental herbarium specimens, this was the sexual state classified in *Capronia, Trichomerium* and some other genera with poor representation in modern sequence-based taxonomy. (2) Species descriptions based on cultured strains started with the classical paper of Schol-Schwarz (1968). In culture, most species exhibit asexual conidia only. Very few authors (Müller et al. 1987) reported sexual and asexual forms of propagation of species, while Untereiner and Naveau (1999) developed a method to promote sexual sporulation in vitro, which was applied to only a limited number of species. The cultured species were therefore classified according to their type of conidiogenesis. With the decrease of novel introductions of sexual species, an increase in conidial species is observed.

(3) Sequence-based taxa mark a third type of species, independent from sexuality. Most types of conidial morphology proved to be polyphyletic, distributed throughout the order. For example, *Cladophialophora* species defined by simple conidial chains can be found scattered across four families/groups: *Epibryaceae, Herpotrichiellaceae, Trichomeriaceae*, and the Domatia clade (Quan et al. 2020). The latest expansion of the described taxonomic novelties disregards forms of sporulation, but applies solely molecular characters. Numbers of novel species descriptions in eight ascosporulating genera (*Chaetothyrium, Ceramothyrium, Capronia, Herpotrichiella, Dictyotrichiella, Trichomerium, Phaeosaccardinula, Epibryon*) were counted in Index Fungorum (www.indexfungorum.org). Before 2000, 255 sexual species have been described, and only 60 sexual species were found after 2000. In contrast, the number of recent novel species in other genera has exploded with 248 introductions after 2000 (Fig. 7). Besides, preferred habitats such as tropical ant-nests have as yet been poorly explored. Irrespective of methodical bias, the order is in an early process of evolutionary diversification (Quan et al. 2020). Consequently, the number of species in the order is likely to expand significantly over the years to come, and the taxonomy of *Chaetothyriales* will remain unstable. We think it is too early to replace the morphological system for a molecular one, since clades are poorly supported and thus molecular genera difficult to define.

Using the records in the taxonomy browser of NCBI as of 23–04-2022, the *Chaetothyriales* comprise a total of 326 sequenced species classified in 46 genera in 6 families, and 24 species defined as *incertae sedis*. In order to obtain a general overview of the order, we collected as many ITS and/or LSU sequences of published species as possible according to the records in GenBank, supplemented with some unpublished sequences of ant-associated species
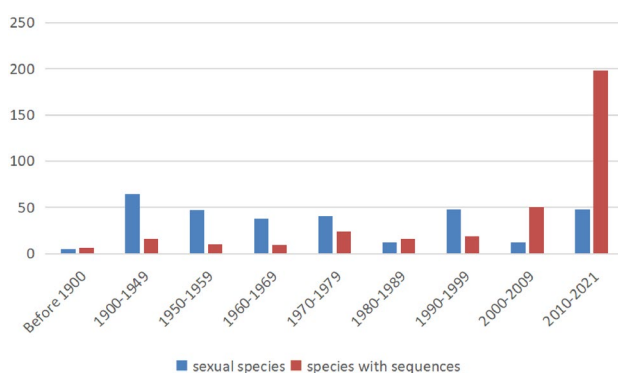

Fig. 7 Sexual species and species with sequences of *Chaetothyriales* described over time. The number of species with sequence increases year by year, especially after 2000, the number of publications of such species increases dramatically. However, no significant increase in the number of sexual species

confirmed to belong to *Chaetothyriales*. A total of 396 sequences were included, of which 321 were of described and 75 of unknown species (Table S1). These published species accounted for 91.71% of all species recorded in NCBI Taxonomy, and hence the tree is regarded to reflect the current taxonomic status of *Chaetothyriales* optimally. Two algorithms, maximum likelihood (ML) and Bayesian inference (BI) were applied to construct the phylogenetic tree, these two types of trees were congruent at family level and most genus level only with little differences in support level (Fig. 8). Both of them proved to be good enough in terms of resolution. Most of the unknown species listed by Quan et al. (2020) were derived from rock and from ant nests, the latter being a recently explored habitat of chaetothyrialean species (Schlick-Steiner et al. 2008; Mayer and Voglmayr 2009; Voglmayr et al. 2011). Strains presented in NCBI lacking ITS or LSU were not included in this study. At present, the tree comprises nine clades, representing the family level, most of them having high bootstrap support. Five clades are consistent with previously described families (Barr 1976, 1987; Chomnunti et al. 2012; Réblová et al. 2013; Gueidan et al. 2014), and two clades deserving family level recognition are still unpublished.

Clade 1 represents the family *Herpotrichiellaceae*, which is the largest family in this order with 180 species, including 14 undescribed species associated with ant carton and one species associated with domatia. An overview of genera based on their type species was given by Moussa et al. (2017). Two genera, i.e. *Bonordeniella* (type species: *B. memorandum*), and *Heteroconium* (type species: *H. cytharexyli*) were recorded to belong to this family but were not included in the tree because of the lack of verified ITS or LSU sequences; their generic status requires verification. Most species of the large genus *Exophiala* (type species *E. salmonis*) clustered in this clade, although a few species were scattered among different clades. For example, *Exophiala encephalarti, E. placitae* and *E. eucalyptorum* were remote from the *Herpotrichiellaceae* clade. The correct name for *Pullularia prototropha* (Kirk et al. 2020) is *Exophiala prototropha. Exophiala calicioides* and *E. lavatrina* were excluded from this study because no correct ITS or LSU are available in NCBI. The taxonomy of another large genus, *Cladophialophora* (type species *C. ajelloi* = *C. carrionii*) also needs adjustment. Because of its simple morphology consisting of dry conidial chains, it is polyphyletic within the order *Chaetothyriales* (Feng et al. 2013). Only relatively few species of the sexual genus *Capronia* [type species *C. sexdecimspora*, which is lost, replaced reference suggested by Quan et al. 2020 is *C. pilosella*] have been studied in culture. All *Capronia* species that have been sequenced to date clustered in Clade 1, except for *C. villosa* which is close to the members of *Epibryaceae*. In the family *Herpotrichiellaceae*, the classical genus *Phialophora* (type species *P. verrucosa*) is problematic. *Phialophora verrucosa*
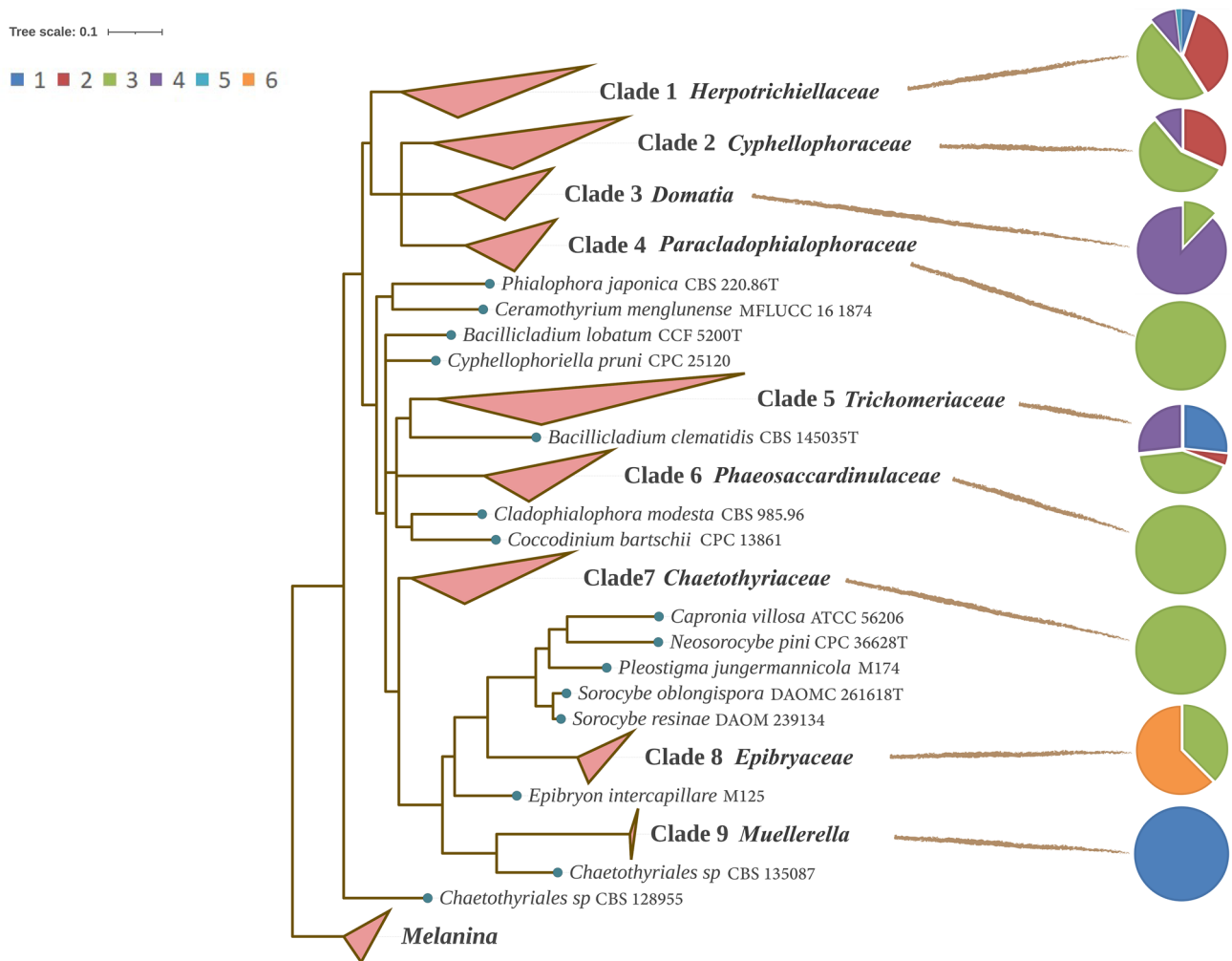
Fig. 8 Combined results of ecology and phylogeny in the order *Chaetothyriales*. Phylogenetic tree (Bayesian) obtained by combined analysis of ITS and LSU sequences of 396 species. Bootstrap values of family clades are shown. Two *Melanina* species were used as an out-group. Different colors of six generalized ecologies were calculated for each clade. 1 Epilithic, 2 opportunistic, 3 epiphytic, 4 myrmecophilic, 5 toxic, 6 bryophytic

clusters with the type of *Cladophialophora, C. carrionii*. Currently, 22 *Phialophora* species have been sequenced, five of which clustered in *Herpotrichiellaceae* and another five were transferred to *Cyphellophora* (family *Cyphellophoraceae*). The remaining twelve species are unrelated and were excluded from this study, continuing reclassifications of phialophora-like species by Gams (2000). The genus *Neosorocybe* (type species *N. pini*) has been mentioned in *Herpotrichiellaceae*, but in this tree, it was at the ancestral clade remote from this family.

Clade 2, the family *Cyphellophoraceae* with 100 (pp) and 89 (BS) support, contains 33 species in 3 genera. All species recorded in NCBI in this family are included in this clade. In addition, the cluster contains five strains from ant carton that were originally identified as *Phialophora* spp.

Clade 3 consists of two subclades. Twenty two sequences in the first subclade are all derived from ant domatia. The second subclade comprised three *Cladophialophora* species from plants without information on the detailed habitat.

Clade 4, two *Paracladophialophora* species form a single clade close to Clade 3. These strains clustered at a long branch, suggesting that the family *Paracladophialophoraceae* described for this group (Crous and Groenewald 2018) is undersampled.

Clade 5 consists of 101 sequences and represents the family *Trichomeriaceae*. Twenty carton strains and six domatia strains are included. These domatia stains did not cluster with the majority of domatia strains which form a separate subcluster mentioned above, but are found adjacent to carton strains. This suggests some resemblance between carton nests and domatia, possibly triggered by ant pheromones (Morgan 2009). According to the taxonomy browser of NCBI, the genus *Metulocladosporiella* contains six species belonging to the family *Herpotrichiellaceae*. However,

our phylogeny of the *Chaetothyriales* places the genus in *Trichomeriaceae,* with 100 bootstrap support. The genus *Bacillicladium* contains two species, which however did not cluster together at long distance, which necessitates re-evaluation of this genus. *Exophiala placitae* and *E. encephalarti* also cluster in *Trichomeriaceae*, which the generic type species is in *Herpotrichiellaceae*, thus requiring reclassification.

Clade 6 contains six sequences of the genus *Phaeosaccardinula*. The genus *Phaeosaccardinula* contains 14 species (Kirk et al. 2008), of which the major part has not been sequenced. Most of these species were isolated from living leaves and are probably phyllosphere fungi. In our study, all the sequences of the genus individualized as a separate clade at 100% bootstrap support. The family *Phaeosaccardinulaceae* (Crous and Groenewald 2018) might be the correct place to accommodate the species. Judging from older records of species described after herbarium material, this part of the tree is severely undersampled.

Clade 7 consists of 24 sequences representing the family *Chaetothyriaceae*, high bootstrap (91) value in the Bayesian tree, but at low bootstrap (40) support with ML. This again suggests severe undersampling of sequence data, compared with the large diversity described on the natural substrate.

Clade 8 represents the family *Epibryaceae*, which consists of ten strains in total. Three of these have been described in *Cladophialophora*, a genus of *Herpotrichiellaceae*, but catenate conidia apparently also occur in cultural states of *Epibryon* species. Hence these species need reclassification.

Two more undescribed clades (**Clades 9 and outgroup**) were noted, all of which were derived from rock environments with non-lichenized, endolichenic lifestyles (Muggia et al. 2021). Clade 9 is the basal clade in the tree, also the oldest clade of *Chaetothyriales*, which we speculate to have coevolved with lichens on rock surfaces. Outgroup clade (= Clade 5 in Quan et al. 2020) was described by Muggia et al. (2021) as the new genus *Melanina*. In the present ordinal overview (Fig. 8), it was selected to root the tree.

Conclusions

The peculiar behaviour of members of *Chaetothyriales* in *Herpotrichiellaceae*, known as 'dual ecology' with a potency of bioremediation as well as infection, seems to have an origin in their early ancestry as endolichenic fungi. This lifestyle has enabled divergent adaptations in chaetothyrialean families, each focusing on aspects of endolichenic life. Among these are extremotolerance on exposed surfaces, ant-association with toxin-tolerance, accumulation in the hydrocarbon-polluted domestic environment via expansion of cytochromes, and endophytism inside plants via microaerophily. In combination, these factors also enhance

survival inside living vertebrate tissue. The fact that, in addition to humans, only cold-blooded vertebrates are infected might be explained by *Chaetothyriales* not being pathogens but opportunists, causing infection almost only via accidental trauma. Nearly all mammals and birds are protected by their fur or feathers, while fish, amphibians and humans have a moist, exposed skin. Once inside the host, *Chaetothyriales* appear recalcitrant and able to sustain over longer periods, often helped by the meristematic growth form that is known from life on rock, i.e., the MC. This cellular form might be sufficiently successful in enhancing survival inside tissue to allow further adaptation towards pathogenicity.

Materials and methods

Strains and sequences

Described species used in this study were selected from NCBI according to the Taxonomy browser. In view of optimal resolution of phylogenetic relationships, only one sequence per species was applied. Up to now (April 2022), GenBank records at NCBI list the order *Chaetothyriales* with 6 families, 45 genera and 325 species, 316 species of them were included in this study, also as 78 undescribed strains and two outgroup species (Table S1). Species not included are either missing sequences for the LSU or ITS or both, or these sequences are obvious errors in NCBI. Undescribed species are mainly inhabitants of ant nests (carton-building ants or domatia) (Voglmayr et al. 2011; Nepel et al. 2014).

Alignment and phylogenetic analysis

Sequences of ITS and LSU of related strains were obtained from NCBI and edited using BIOEDIT v7.2 (Hall 1999). Alignments were made by MAFFT v7 (http://mafft.cbrc.jp/) and optimized manually using MEGA v7.2 (Kumar et al. 2012) and BIOEDIT v7.2. Missing data for partial or complete sequences in some taxa were coded as 'missing' (Wiens 2006). To address the phylogenetic relationships among taxa, ML and BI algorithms were used. Two *Melanina* species were taken as outgroups. The ML tree was obtained using RAxML-VI-HPC as implemented on the CIPRES portal web server (http://www.phylo.org/). Bayesian command files were prepared using MESQUITE v2.75 (Maddison and Maddison 2007), and the analysis was done in MRBAYES v3.1.2 implemented in the CIPRES web server. Two parallel runs with four Markov chain Monte Carlo (MCMC) simulations for each run were set for 20,000,000 generations and the result was checked using TRACER v1.5 (Rambaut and Drummond 2009) for effective sample size (ESS). The run was then extended for another

10,000,000 generations with a sample frequency of 1000 per generation. Trees were edited using Treeview v1.6.6 and completed with Adobe ILLUSTRATOR CS v5.

Genome assembly and annotation

A total of 44 species are involved in the genomic analysis, most of them being obtained from NCBI and published in earlier studies. Metadata of species included in the study are provided in Table S2. Genomic DNA of *Camptophora hylomeconis* (CBS 113311), *Epibryon bryophilum* (CBS 126278) and *Strelitziana albiziae* (CBS 126497) was extracted from cell pellets harvested from cultures incubated for 14 days at 28 °C with Fungi DNA Kit (Omega Bio-Tek, Norcross, GA, USA) according to the manufacturer's instructions. DNA concentration was quantified using a TBS-380 fluorometer (Turner BioSystems, Sunnyvale, CA, USA). Highly qualified DNA samples (OD260/280 = 1.8–2.0, > 6 μg) were utilized to construct a fragment library with 400 bp insert size. Sequencing was performed at Eurofins (Hamburg, Germany) on an Illumina Hiseq. The quality control of the reads was performed using FastQC v0.11.8 (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/) and the low-quality sequences were removed by Trimmomatic v0.39 (http://www.usadellab.org/cms/?page=trimmomatic). The reads were assembled by Megahit v1.2.9 (Li et al. 2015a, b) and subsequently by SPADES v3.15.5 (Bankevich et al. 2012). QUAST v4.6 (http://quast.sourceforge.net/) was used to access assembly quality as well as BUSCO (Simão et al. 2015), using the chaetothyriales_odb10 dataset, to evaluate genome completeness. Gene prediction and annotation was done using Funannotate v1.7.0 (https://funannotate.readthedocs.io/en/latest/index.html), which includes InterProScan and EggnogMapper.

Declarations

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