Black fungi and ants: a genomic comparison of species inhabiting carton nests versus domatia

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Abstract
Some members of Chaetothyriales, an order containing potential agents of opportunistic infections in humans, have a natural habitat in nests of tropical arboreal ants. In these black fungi, two types of ant symbiosis are known, i.e. occurrence in domatia inside living plants, or as components of carton constructions made of ant-chewed plant tissue. In order to explain differences between strains from these types of association, we sequenced and annotated genomes of two newly described carton species, Incumbomyces lentus and Incumbomyces delicatus, and compared these with genomes of four domatia species and related Chaetothyriales. General genomic characteristics, CYP genes, carbohydrate-active enzymes (CAZymes), secondary metabolism, and sex-related genes were included in the study.

Keywords: Black fungi, Carton fungi, Chaetothyriales, Comparative genomics

Introduction
Ants (Arthropoda: Formicidae) are ubiquitous in nearly all climate zones worldwide and play important roles in many ecosystems. The sum of ant bodies has been estimated to account for 15–20% of terrestrial animal biomass (Schultz 2000). Interactions between ants and fungi were already recorded more than 100 years ago (Bailey 1920; Huxley 1978; Miehe 1911). These not only concern the Attine-fungi mutualistic symbioses in general in fungus gardens (Mueller et al. 2018), but a wide diversity of less pronounced interactions occur (Biedermann and Vega 2020; Moreau 2020). Among the black fungi, Cladosporium myrmecophilum is a classical colonizer of debris in carton nests of Lasius fuliginosus (Schlick-Steiner et al. 2008). Fungi identified with sequence data as belonging to the order Chaetothyriales were found a decade ago, when phialophora-like species were recurrently isolated from ants and their constructions (Little and Currie 2007; Defossez et al. 2009; Mayer et al. 2018). Many of these fungi seem to have ant-association as their main habitat choice, and many are new to science. The discovery and full description of these new species will have a profound impact on the current classification of Chaetothyriales.

The order Chaetothyriales (Ascomycota) is particularly known through the black yeasts and filamentous relatives that cause opportunistic infections in humans. The species in this order tend to be abundant in extreme or tannin-rich natural habitats. The best-known species, however, are the ones that are found in domesticated environments that are rich in creosote or toxic hydrocarbons (Dögen et al. 2013) or in habitats with high...
temperature and/or poor in nutrients (Gostinčar et al. 2012). This extremotolerance has been hypothesized to contribute to invasive abilities of sterile sites of the vertebrate body (Quan et al. 2020a, b). As ants produce and communicate with ketones and low-molecular hydrocarbons (Di Mauro et al. 2015) which also function as antimicrobial compounds in ant nests and constructions, the ants might have played a role in early evolution of extremophily observed in Chaetothyriales. While nine clades of the order have been redefined to family level (Quan et al. 2020a, b), the majority of species with close vicinity to ants, i.e. those residing in their nesting space provided by their host-plant (= domatia), clustered in a single, as yet poorly defined clade, which might deserve family status. Some additional domatia-associated species are found in the Trichomeriaceae. Species associated with carton exposed to the environment seem less specialized and are scattered in three families, i.e. Cyphellophoracere, Herpotrichiellaceae, and Trichomeriaceae (Voglmayr et al. 2011; Vasse et al. 2017). Morphologically, Incumbomyces species, as other carton strains, lack conidiation in culture, while domatia-fungi show a consistent type of sympodial propagation with mucous conidia (Voglmayr et al. 2011; Quan et al. 2020a, b). The morphological differences might be related to vectors of dispersal e.g. with ant bodies.

The deviating morphology and phylogenies of carton- versus domatia-associated black fungi suggests that Chaetothyriales have gone through different types of evolution with the ant as driver. Domatia are modified leaves, stems or roots that provide cavities occupied by ants, offered by about 680 species of tropical vascular plants. Once inhabited by ants, the domatia contain dark patches from which black fungi can be isolated (Nepel et al. 2016). Recent data show that colony-founding queens start growing chaetothyrialean fungi in the domatia already before they lay their eggs, and that the queens do not feed on fungal material themselves but feed it to the larvae (Mayer et al. 2018). The second type of association of ants and Chaetothyriales is referred to as carton nesting. Nests consist of chewed plant material and have a cardboard-like appearance. Fungal hyphae are abundantly found in the walls of nests and galleries and strengthen the constructions. The main function of the carton structure is to increase the space of the nest and to enhance defense strategies (Vasse et al. 2017). The fungi in these structures do not serve as food, but increase stability of the nests (Hölldobler and Wilson 1990).

Genome analysis and comparison is a widely used biotechnology and may provide clues towards understanding of microbial ecology. At present, 45 genomes of Chaetothyriales have been published. Teixeira et al. (2017) analyzed 23 genomes of Chaetothyriales and provided a detailed comparative analysis, revealing genes related to protein degradation, carbohydrate-active enzymes (CAZymes), melanin synthesis and secondary metabolism. Moreno et al. (2019) was the first to analyze genomes of Chaetothyriales from ant domatia. The domatia-associated species were found to have remarkably small genomes, low amounts of protein-coding genes, and a high degree of repetitive elements. In addition, the proportion of biosynthetic clusters involved in the production of secondary metabolites and potential antibacterial activities were overrepresented. Attili-Angelis et al. (2014) described some species from ant bodies rather than from nesting material; these genomes represent members of Cyphellophoraceae.

Two novel genomes in the present study were derived from CBS 128958 and CBS 129047, recently described as types of Incumbomyces lentus and I. delicatus, respectively, in the family Trichomeriaceae. Both originated from ant carton structures of ant species in tropical Southeast Asia structures (Voglmayr et al. 2011). Although carton fungi and domatia fungi both belong to Chaetothyriales, their positions within the order are markedly different. Habitat conditions within domatia are balanced, whereas carton structures are subjected to fluctuating environmental conditions. The difference in habitat choices of the black fungi may have had profound evolutionary consequences. The aim of the present paper is to compare some parameters which may have played a role in these differential phylogenies.

Materials and methods

Strains and sequencing

Genomic DNA of Incumbomyces lentus CBS 128958, isolated from carton of a Monomorium sp. ant nest in Malaysia and I. delicatus CBS 129047, isolated from carton of a Crematogaster sp. ant nest in Thailand (Quan et al. 2020a, b) was extracted from cell pellets harvested from cultures incubated for 14 days at 28 °C with Fungi DNA Kit (Omega Bio-Tek, Norcross, GA, U.S.A.) according to the manufacturer’s instructions. DNA concentration was quantified using a TBS-380 fluorometer (Turner BioSystems, Sunnyvale, CA, U.S.A.). High 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 Biozeron Biotechnology Company (Shanghai, China) on Illumina Hiseq and Pacific Bioscience platforms. Information of species included in the study is provided in Additional file 1: Table S1.

Alignment and phylogenetic analysis

The combined sequences of ITS and LSU of Chaetothyriales species in this study 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, Maximum Likelihood (ML) was used. Species of Melanina 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/). The tree was edited using TREEVIEW v1.6.6 and completed with Adobe ILLUSTRATOR CS v5.

Results
Phylogeny
The phylogenetic tree contains a total of 255 sequences including four of Melanina as outgroup (Additional file 2: Table S2). Two carton species, four domatia strains and 249 remaining Chaetothyriales species were analyzed. Throughout the tree, eight clades described at family level were recognized. Bootstrap values of each family are showed in Fig. 1. Carton strains are scattered throughout the tree, clustering in three families: Herpotrichiellaceae, Cyphellophoraceae and Trichomeriaceae. Most domatia strains clustered in a single clade together with two Cladophialophora species. A few domatia strains clustered in the family Trichomeriaceae, close to carton strains which are rather commonly found in the same family.

Gene assembly and gene prediction
Genome sequences of two Incumbomyces species originating from carton of ant nests were determined by Illumina and Pacific Bioscience and de novo assembled. After cleaning, a total of 27.1 Mb and 31.5 Mb high quality reads were generated for the strains CBS 128958 and CBS 129047, I. lentus and I. delicatus, respectively. GC contents were 50.78% (I. lentus) and 49.37% (I. delicatus), which is similar to most other black yeast species (Teixeira et al. 2017). Protein-coding gene compositions were determined by ab initio gene prediction methods. The highest gene count of 9530 genes was found in I. delicatus, which also had the largest genome of all ant-associated species investigated (31.5 Mb), whereas the domatia-associated strain CBS 134916 comprised only 6719 genes (Moreno et al. 2019). I. lentus was intermediate in having 8868 genes, similar to the species Arthrocladium fulminans (8534 genes), which belongs to Trichomeriaceae, i.e., the same family as carton fungi in Incumbomyces. Both average gene lengths of carton fungi (in bp) were similar, i.e., 1645 and 1637 bp. The number of tRNAs were different between the two studied strains. Numbers of tRNAs of carton strains (143–208) were much higher than those in domatia-associated strains (37–76). The contents of repetitive elements in I. lentus (CBS 128958) and I. delicatus (CBS 129047) were 1.75% and 3.41%, respectively, which was on average significantly higher than in other thus far sequenced species of Chaetothyriales without ant-related ecology (ranging from 0.03 to 5.2%; Teixeira et al. 2017). In Arthrocladium fulminans, the repetitive elements comprised 1.75%, while in domatia fungi percentages were very high, ranging from 4.09 to 16.32%. All basic information of carton species, domatia species and Arthrocladium fulminans is listed in Table 1. Using ORTHOVEN2, we determined the core gene clusters that were conserved in both carton and
Fig. 1  Phylogenetic tree of Chaetothyriales based on ITS and LSU sequences, obtained by maximum likelihood. Four Melanina strains were used as outgroup. Green dots represent domatia fungi and black dots represent carton fungi.
domatia fungi, and in other species of Chaetothyriales. A range from 5586 to 13,269 orthologous clusters was detected among the 26 studied isolates, including carton fungi and domatia fungi (Fig. 2, Table 2). This resulted in 3096 clusters per genome in the core set conserved in all isolates. Among these, the average number of unique
genes was 314 existing in the two carton fungi, while the average unique genes in domatia species only was 67, and 188 clusters were represented in *A. fulminans*.

**Carbohydrate-active enzymes (CAZymes)**

CAZymes are responsible for the degradation, modification, and biosynthesis of carbohydrates and glycoconjugates (Cantarel et al. 2009). Based on amino-acid sequence and structure similarity, the CAZymes can be classified into five classes of enzyme activities and one associated module: glycoside hydrolases (GHs), glycosyl transferases (GTs), polysaccharide lyases (PLs), carbohydrate esterases (CEs), auxiliary activities (AAs), and the associated module carbohydrate-binding modules (CBMs) (Cantarel et al. 2009). The total numbers of genes in CAZymes families of *I. lentus* and *I. delicatus* are 288 and 293, respectively. This is higher than found in domatia fungi (ranging from 208 to 233), but comparable to members of Herpotrichiellaceae (ranging from 285 to 434) and *A. fulminans* in Trichomeriaceae (269) (Table 3).

Polysaccharide lyases (PLs) are a group of enzymes that cleave uronic acid-containing polysaccharide chains via a β-elimination mechanism to generate an unsaturated hexenuronic acid residue and a new reducing end. Both *Incumbomyces* carton strains possess the polysaccharide lyase subfamily 3 (PL3); the two domatia strains (CBS 135957 and CBS 134920) possess subfamily 4 (PL1), while two domatia strains (CBS 134916 and CBS 132003) were similar to most other Chaetothyriales, lacking pectinases; PL3 was so far detected only in *Capronia coronata* (Herpotrichiellaceae), a species originating from decoricated wood, and in *Cyphellophora attinorum* (Cyphellophoraceae), which was isolated from the cuticle of tropical ant gynes (Additional file 3: Table S3).

Glycoside hydrolases (GHs) are a widespread group of enzymes which hydrolyze the glycosidic bond between two or more carbohydrates or between a carbohydrate and a non-carbohydrate moiety. The number of GH

### Table 2 The number of orthology classes in ant-associated fungi and other Chaetothyriales fungi, including core genes, shared genes, unique gene

| Species                   | Number of genes in orthogroups | Core all species | Shared genes | Number of specific genes |
|---------------------------|---------------------------------|-----------------|--------------|-------------------------|
| *Incumbomyces lentus*     | 9570                            | 3096            | 6214         | 260                     |
| *Incumbomyces delicatus*  | 10,228                          | 3096            | 6765         | 367                     |
| *Arthrocladium fulminans* | 8346                            | 3096            | 5062         | 188                     |
| *Chaetothyriales sp. CBS 134,920* | 5916                              | 3096            | 2752         | 68                      |
| *Chaetothyriales sp. CBS 135,597* | 5586                              | 3096            | 2454         | 36                      |
| *Chaetothyriales sp. CBS 134,916* | 6605                              | 3096            | 3411         | 98                      |
| *Chaetothyriales sp. CBS 132,003* | 5662                              | 3096            | 2501         | 67                      |
| *Phialophora attinorum*   | 10,935                          | 3096            | 6926         | 913                     |
| *Cyphellophora europaea*  | 10,622                          | 3096            | 7054         | 472                     |
| *Exophiala dermatitidis*  | 9117                            | 3096            | 5560         | 461                     |
| *Exophiala sderis*        | 10,650                          | 3096            | 7084         | 470                     |
| *Exophiala xenobiatica*   | 12,696                          | 3096            | 9109         | 491                     |
| *Exophiala oligosperma*   | 12,642                          | 3096            | 8954         | 592                     |
| *Exophiala spinifera*     | 11,783                          | 3096            | 8421         | 266                     |
| *Exophiala aquamarina*    | 12,839                          | 3096            | 9464         | 279                     |
| *Exophiala mesophila*     | 9977                            | 3096            | 6511         | 370                     |
| *Rhinocladiella mackenziei* | 10,997                         | 3096            | 7516         | 385                     |
| *Capronia coronata*      | 9140                            | 3096            | 5953         | 91                      |
| *Cladophialophora psammophila* | 13,227                           | 3096            | 9937         | 194                     |
| *Cladophialophora bantiana* | 12,512                          | 3096            | 9166         | 250                     |
| *Fonsecaea multimorphosa* | 12,173                          | 3096            | 8881         | 196                     |
| *Cladophialophora immunda* | 13,269                          | 3096            | 9409         | 764                     |
| *Fonsecaea pedrosii*     | 12,245                          | 3096            | 8867         | 282                     |
| *Cladophialophora yegresii* | 10,023                          | 3096            | 6832         | 95                      |
| *Cladophialophora carrioni* | 10,478                          | 3096            | 6679         | 703                     |
| *Phialophora americana*  | 11,507                          | 3096            | 8009         | 402                     |
families of the carton fungi are 128 and 135, more than that present in domatia fungi (85–96; Table 3). GH families that exist in carton fungi but are absent from domatia fungi are GH27, GH36, GH79, and GH92. GH 27 and GH 36 mainly associated with galactosidase-related enzymes. GH 79 associated with glucuronidase-related enzymes, and GH92 are mainly focused on mannosidase.

Glycosyltransferases (GTs) are enzymes that catalyze the transfer of sugar moieties from activated donor molecules to specific acceptor molecules, forming glycosidic bonds. On average, members of Herpotrichiellaceae contain somewhat larger numbers (76–123, av. 93.9) than remaining members of Chaetothyriales (62–98, av. 78.7). A similar difference was observed with carbohydrate esterases (CEs), members of Herpotrichiellaceae having 39–84 (av. 61.7), against 26–69 (av. 39.8) for remaining species. Exophiala dermatitidis was consistently exceptional in Herpotrichiellaceae, having genome size and gene numbers like Incumbomyces species (Table 3).

The AA class presently groups nine families of ligninolytic enzymes and 6 families of lytic polysaccharide mono-oxygenases. A notable difference was observed in the numbers of AA7 of carton fungi being 14, while only 3–7 AA7 were present in domatia fungi.

Cytochrome p450 genes (CYPs)
Cytochrome p450 genes exist widely in all kingdoms and play important roles in primary and secondary metabolism, and in drug and xenobiotic resistance. Our study used CYPminer, an automatic tool that allows processing of large numbers of sequence data to identify and classify CYPs. The identification results of CYPminer are more detailed and abundant than with use of the previous database, the PFAM protein family database. Analyzing

| Species                       | GH  | GT  | CE  | AA  | CBM | PL  | Total |
|-------------------------------|-----|-----|-----|-----|-----|-----|-------|
| **Trichomeriaceae**           |     |     |     |     |     |     |       |
| Incumbomyces lentus           | 128 | 81  | 37  | 40  | 1   | 1   | 288   |
| Incumbomyces delicatus        | 135 | 79  | 38  | 39  | 1   | 1   | 293   |
| Arthrocladium fulminans       | 135 | 62  | 39  | 31  | 1   | 1   | 269   |
| **Domatia**                   |     |     |     |     |     |     |       |
| Chaetothyriales sp. CBS 134920| 87  | 74  | 30  | 30  | 1   | 1   | 223   |
| Chaetothyriales sp. CBS 135597| 85  | 74  | 26  | 33  | 1   | 1   | 220   |
| Chaetothyriales sp. CBS 134916| 96  | 76  | 30  | 30  | 1   | 0   | 233   |
| Chaetothyriales sp. CBS 132003| 86  | 68  | 27  | 26  | 1   | 0   | 208   |
| **Cyphellophoraceae**         |     |     |     |     |     |     |       |
| Phialophora attinorum         | 151 | 98  | 69  | 66  | 3   | 1   | 388   |
| Cyphellophora europaea        | 173 | 96  | 62  | 72  | 3   | 1   | 407   |
| Exophiala dermatitidis        | 118 | 87  | 39  | 37  | 2   | 2   | 285   |
| **Herpotrichiellaceae**       |     |     |     |     |     |     |       |
| Exophiala xenobiotica         | 181 | 110 | 70  | 67  | 4   | 2   | 434   |
| Exophiala oligosperma         | 161 | 123 | 73  | 61  | 3   | 0   | 421   |
| Exophiala spinifera           | 146 | 106 | 71  | 50  | 2   | 1   | 376   |
| Exophiala aquamarina          | 162 | 96  | 84  | 63  | 4   | 0   | 409   |
| Exophiala mesophila           | 118 | 102 | 49  | 52  | 1   | 0   | 322   |
| Rhinocladiella mackenzi 8     | 119 | 76  | 53  | 58  | 4   | 1   | 311   |
| Capronia coronata             | 112 | 84  | 52  | 34  | 3   | 2   | 287   |
| Capronia epimyces             | 104 | 89  | 61  | 44  | 1   | 0   | 299   |
| Cladophialophora psammophila  | 141 | 91  | 78  | 77  | 3   | 0   | 390   |
| Cladophialophora bantiana     | 143 | 90  | 66  | 70  | 3   | 0   | 372   |
| Fonsecaea multiformosa        | 140 | 90  | 68  | 77  | 2   | 0   | 377   |
| Cladophialophora immunda      | 148 | 101 | 64  | 71  | 2   | 0   | 386   |
| Fonsecaea pedrosii            | 133 | 89  | 81  | 65  | 2   | 0   | 370   |
| Cladophialophora yegresii     | 129 | 82  | 46  | 48  | 2   | 0   | 307   |
| Cladophialophora carrionii    | 137 | 87  | 43  | 50  | 3   | 1   | 321   |
| Capronia semimmersa           | 140 | 94  | 51  | 54  | 3   | 0   | 342   |

GH: glycoside hydrolases, GT: glycosyl transferases, CE: carbohydrate esterases, AA: auxiliary activities, CBM: associated carbohydrate-binding modules, PL: polysaccharide lyases.
of the same family as *Incumbomyces*, Trichomeriaceae, and solely known from an infection in a human patient. *A. fulminans* has four more CYP families (CYP5199, CYP570, CYP619, CYP60) compared to domatia species; the remaining CYPs are shared. Seven CYPs (CYP5076, CYP5077, CYP5081, CYP5282, CYP5295, CYP5307, CYP631) were found only in domatia and were lacking in other members of Chaetothyriales. Most of these genes are related to xenobiotic or secondary metabolism (Chadha et al. 2018).

### Secondary metabolism

Secondary metabolism refers to the metabolic pathways and small molecular products involved in ecological interaction, which are not essential for the survival of the organism but may be significant, e.g. for the colonization of a specific niche. Summarizing the wide diversity of secondary metabolites, the following broad types can be distinguished according to enzymes involved in synthetic pathways: polyketides (PKS), non-ribosomal peptides (NRPS), and terpenes and indole alkaloids (Keller et al. 2005). Several of the sequenced fungal genomes contained hybrid genes, such as PKS-NRPS. *Incumbomyces delicatus* and *I. lentus* possess 7 and 8 biosynthetic clusters, respectively (Fig. 3, Table 5). The NRPS type is prevalent in clusters of both carton species. The type III PKS cluster (t3 PKS), previously reported in Herpotrichiellaceae, Cyphellophoraceae, and Trichomeriaceae is absent from carton and domatia species. In addition, both species had only one or two 1 PKS, which is far less

### Table 4 Distribution of CYP p450 genes in in ant-associated fungi and other Chaetothyriales fungi

| Species                     | Ecology    | Genes of family | Genes of new family | Genes of total family | Genes of subfamily | Genes of new subfamily | Total genes |
|-----------------------------|------------|-----------------|---------------------|-----------------------|--------------------|------------------------|-------------|
| **Trichomeriaceae**         |            |                 |                     |                       |                    |                        |             |
| *Incumbomyces lentus*       | Carton     | 58              | 5                   | 63                    | 15                 | 42                     | 105         |
| *Incumbomyces delicatus*    | Carton     | 62              | 5                   | 67                    | 17                 | 44                     | 111         |
| *Arthrocladium fulminans*   | Patient    | 66              | 6                   | 72                    | 20                 | 45                     | 117         |
| **Domatia**                 |            |                 |                     |                       |                    |                        |             |
| Chaetothyriales sp. CBS 135597 | Domatia  | 74              | 9                   | 83                    | 27                 | 56                     | 139         |
| Chaetothyriales sp. CBS 134916 | Domatia  | 68              | 6                   | 74                    | 19                 | 54                     | 128         |
| **Cyphellophoraceae**       |            |                 |                     |                       |                    |                        |             |
| *Cyphellophora attinorum*   | Ant        | 116             | 9                   | 125                   | 26                 | 88                     | 213         |
| **Herpotrichiellaceae**     |            |                 |                     |                       |                    |                        |             |
| *Exophiala xenobiotica*     | Oil sludge | 142             | 18                  | 160                   | 33                 | 108                    | 268         |
| *Exophiala oligosperma*     | Patient    | 116             | 20                  | 136                   | 28                 | 94                     | 230         |
| *Rhinocladiella macrospora* | Patient    | 135             | 25                  | 160                   | 35                 | 109                    | 269         |
| *Capronia coronata*         | Wood       | 81              | 9                   | 90                    | 29                 | 61                     | 151         |
| *Cladophialophora psammophila* | Polluted soil | 178            | 18                  | 196                   | 44                 | 126                    | 322         |
| *Cladophialophora bracteata* | Patient    | 139             | 16                  | 155                   | 35                 | 99                     | 254         |
| *Fonseca pedrosi*           | Patient    | 142             | 11                  | 153                   | 49                 | 44                     | 197         |
than observed in domatia strains (11 on average). The number of terpenes in *Incumbomyces* was also far less than in remaining Chaetothyriales (Teixeira et al. 2017).

### Asexual and sexual reproduction

The mating type locus is a unique region in the fungal genome, playing a central role in the sexual cycle. We identified the mating type idiomorph, flanking genes and heterokaryon incompatibility protein (HET-PF06985) in the genomes of *Incumbomyces* carton fungi and compared these with four domatia species and other members of Chaetothyriales described previously. In none of the *Incumbomyces* species, sporulation has been observed, neither sexual nor clonal (Quan et al. 2020a, b). *I. delicatus* (CBS 129047) contained a single mating type (MAT1-2) and thus was found to be heterothallic. In contrast, *I. lentus* (CBS 128958) was confirmed to have both MAT1-1 and MAT1-2, which were closely clustered in a single assembled scaffold; hence *Incumbomyces lentus* was homothallic (Fig. 4).

#### Table 5  Summary of secondary-metabolite gene classes in ant-associated fungi

| Species                  | Terpene | III PKS | I PKS | III PKS/I PKS | NRPS | I PKS/NRPS |
|--------------------------|---------|---------|-------|--------------|------|------------|
| *Incumbomyces lentus*    | 1       | 0       | 2     | 0            | 5    | 0          |
| *Incumbomyces delicatus* | 1       | 0       | 1     | 0            | 5    | 0          |
| Chaetothyriales sp CBS 134916 | 3 | 0       | 8     | 0            | 5    | 0          |
| Chaetothyriales sp CBS 132003 | 3 | 0       | 11    | 0            | 3    | 0          |
| Chaetothyriales sp CBS 134920 | 2 | 0       | 11    | 0            | 6    | 1          |
| Chaetothyriales sp CBS 135597 | 1 | 0       | 12    | 0            | 8    | 1          |

Fig. 3  The region and of amount of secondary-metabolite gene classes in ant-associated fungi
We confirmed the MAT flanking genes APN2, APC5, SLA2 and COX13, which are highly conserved and are distributed widely in members of Eurotiomycetes (Coppin et al. 1997; Fraser et al. 2007; Paoletti et al. 2007). Nevertheless, significant differences in the flanking structure were observed between the two Incumbomyces species. In I. lentus CBS 128958, the APN2, COX13, and APC5 genes were in synteny in the right flanking region, while in I. delicatus CBS 129047 they were in the left flanking region, in inversed order. The SLA2 genes of these two species were not close to other flanking genes. Concerning I. lentus, the MAT locus is localized in scaffold 5 (SLA2 in scaffold 2). In I. delicatus, the MAT locus is localized in scaffold 8 (SLA2 in scaffold 6). Both carton species have the COX13 genes, which are absent from most Chaetothyriales but present in domatia fungi and in members of Trichomeriaceae, i.e. in Incumbomyces and in A. fulminans. The heterokaryon incompatibility protein (HET) ranged from 73 in I. lentus to 105 in I. delicatus. In contrast, the number in domatia strains ranged from 10 in CBS 134920 to 31 in CBS 134916. In other species of Chaetothyriales, the number was extremely variable, ranging from 1 to 134 (Additional file 5: Table S5).

Enzymes representing specific types of ecology

To better understand the ecology of carton fungi and other Chaetothyriales, we selected five types of enzymes, representing specific types of ecology, and listed the number of genes in each category, such as cellulases and pectinases (plant-associated compounds), lipases (animal-associated compounds), chitinases (potentially involved in decomposition of chitinous ant bodies), and ligninolytic enzymes (involved in wood degradation). An observation which is difficult to explain is that A. fulminans, only known from human infection, is unique in Chaetothyriales by having 12 pectinase genes (Table 6).

Discussion

The genus Incumbomyces, containing two described species isolated from walls of ant-made carton nests (Quan et al. 2021), clusters phylogenetically among members of Trichomeriaceae, as a sister group of Bradymyces. It may be questioned whether the genus shows highest similarity with its family members due to shared phylogeny, or with ant-associated Chaetothyriales sharing a similar habitat. The only member of the Trichomeriaceae for which a genome has been sequenced is Arthrocladium fulminans (Moreno et al. 2020). This species is rather exceptional in Trichomeriaceae, being known from two isolates that caused severe infections in humans (Diallo et al. 2017; Egenlauf et al. 2019), while most members of the family comprise surface-colonizing ‘sooty moulds’ (Chomnunti et al. 2012). In addition to this life style, a significant number of ant-associated Chaetothyriales—both carton- and domatia-associated—clustered in the family (Fig. 1). Another group combining oligotrophic surface colonization with pathogenicity is Bradymyces, where an infection in fish was described (Hubka et al. 2014). An interesting analogy of oligotrophy and infectious ability was published by Moreno et al. (2019), hypothesizing an environmental habitat of the neuroinvasive species Rhinocladiella mackenziei in the hydrocarbon-polluted desert. The expanded cytochromes may be instrumental in these seemingly very different life styles. In addition to cytochromes, the species also had choline permease.
which assists in conversion to the osmoprotectant glycine betaine, enhancing survival in the desert. Numerous members of Chaetothyriales, particularly those in Herpotrichiellaceae and scattered in other families, are environmental but predisposed for infection by their melanin, extremotolerance and hydrocarbon assimilation, being pronounced opportunists. The evolution of genes involved in colonization of these habitats deserves attention. Ant domatia are rich in low-molecular volatiles with low toxicity (Prenafeta et al. 2021), which may have been an evolutionary starting point, but more data are needed from comparable species in Trichomeriaceae.

For some general genomic characters, such as genome size, GC content, and gene number, the carton fungi in Incumbomyces proved to be similar to A. fulminans. The domatia fungi (“domata-group” in Fig. 1) differ significantly from all remaining Chaetothyriales known thus far by having small genomes containing a limited number of genes (Moreno et al. 2019; Table 1). The GH families i.e. GH27, GH36, GH79, and GH92 are mostly linked to decomposition of polysaccharides in plant cell walls. Ant nests and tunnels inhabited by Incumbomyces species consist of wood pulp that has been chewed by the ants, whereby the fungal hyphae enhance constructive strength. A CAZy difference between carton and domatia concerns AA7 enzymes in AA class. The amount of these enzymes in carton is much more than that in domatia. Known AA7 enzymes are potentially involved in the biotransformation or detoxification of lignocellulosic compounds (Levasseur et al. 2013). Carton also consists of ant-chewed wood and other plant material, while this material is absent from domatia. Indeed, the total number of CAZymes in carton fungi is higher than that in domatia fungi, indicating that carton fungi have more enzymes to decompose plant material and to synthesize fungal cell components. The habitat of ant debris inside domatia apparently does not require these enzymes.

Ants secrete pheromones such as low-molecular hydrocarbons and ketones (David Morgan 2009) to protect the brood against fungal and bacterial pathogens. Many fungi can be isolated from the nests as their propagules are easily trapped in the complex networks, but few are able to grow in this environment. However, members of Chaetothyriales seem relatively resistant

| Table 6 | The number of five types of enzymes, representing specific types of ecology in each category |
|---------|-------------------------------------------------|
| Species                          | Strain number | Cellulases | Lipases | Chitinases | Ligninolytic | Pectinases |
| Incumbomyces lentus               | CBS 128958    | 7          | 22      | 3          | 8            | 0         |
| Incumbomyces delicatus           | CBS 129047    | 8          | 20      | 5          | 8            | 0         |
| Anthracodium fulminans           | CBS 136243    | 11         | 44      | 28         | 33           | 12        |
| Chaetothyriales sp.              | CBS 135597    | 9          | 16      | 10         | 7            | 0         |
| Chaetothyriales sp.              | CBS 134916    | 11         | 15      | 5          | 9            | 0         |
| Phialophora attinorum            | CBS 131958    | 15         | 53      | 6          | 9            | 0         |
| Phialophora europaea             | CBS 101466    | 17         | 32      | 7          | 11           | 0         |
| Exophiala dermatitidis           | NIH 8656      | 9          | 21      | 5          | 7            | 0         |
| Exophiala sidensis               | CBS 121828    | 11         | 27      | 5          | 7            | 0         |
| Exophiala xenobiotica            | CBS 118157    | 13         | 34      | 7          | 10           | 0         |
| Exophiala oligosperma            | CBS 725.88    | 8          | 36      | 6          | 10           | 0         |
| Exophiala spinifera              | CBS 89968     | 8          | 43      | 5          | 9            | 0         |
| Exophiala aquamarina             | CBS 119918    | 13         | 50      | 6          | 7            | 0         |
| Exophiala mesophila              | CBS 402.95    | 11         | 16      | 4          | 9            | 0         |
| Rhinocladiella mackenzii         | CBS 660.93    | 11         | 27      | 2          | 6            | 0         |
| Capronia coronata                | CBS 617.96    | 11         | 21      | 5          | 8            | 0         |
| Cladophialophora psammophila     | CBS 110553    | 12         | 40      | 4          | 12           | 0         |
| Cladophialophora bantiana        | CBS 173.52    | 11         | 35      | 4          | 12           | 0         |
| Fonsecaea multiforma             | CBS 102226    | 12         | 31      | 3          | 10           | 0         |
| Cladophialophora immunda         | CBS 834.96    | 11         | 24      | 3          | 13           | 0         |
| Fonsecaea pedrosoi               | CBS 271.37    | 10         | 29      | 4          | 11           | 0         |
| Cladophialophora yegresii        | CBS 114405    | 14         | 28      | 3          | 9            | 0         |
| Cladophialophora carrioni        | CBS 160.54    | 13         | 30      | 3          | 8            | 0         |
| Phialophora americana            | CBS 27337     | 14         | 38      | 3          | 8            | 0         |

Cellulases representing the ecology related to plant-associated compounds, lipases representing the ecology related to animal-associated compounds, chitinases potentially involved in decomposition of chitinous ant bodies, ligninolytic enzymes involved in wood degradation
to these compounds, allowing them to colonize the somewhat toxic ant constructions (Mayer et al. 2018). Teixeira et al. (2017) observed cytochrome (CYP) family expansion in Chaetothyriales. The authors actually only analyzed members of Herpotrichiellaceae, a family with a strong association with domestic environments polluted with aromatic hydrocarbons (Isola et al. 2013). CYP expansion is consistent with this habitat. For example, CYP 530 is thought to participate in the degradation of several fatty acids and hydrocarbons. It was found with 12 copies in Cladophialaphora psammophila, a species known from a single isolate from hydrocarbon-polluted soil. This CYP was not found in any of the carton or domatia strains, suggesting that the fungi, reside in a lower-toxic habitat. This absence may be evolutionarily ancestral, as chaetothyrialean members in the derived family Herpotrichiellaceae that survive in hydrocarbon-polluted domesticated environments have shown significant expansion of CYP genes (Teixeira et al. 2017).

Very large differences related to secondary metabolism were noted between species, even among species sharing a similar ecology. Carton and domatia fungi are associated to ants, the enzymes related to the secondary metabolism of both fungi are very different. Particularly the differences in PKS and NRPS are significant (Fig. 3). The exact ecological meaning of these differences is as yet unclear and needs comparison with additional members of Trichomeriaceae from other habitats.

Similar to most species of Trichomeriaceae, sporulation in vitro is nearly absent from cultured Incumbomyces species. Only a few species are known by their Trichomerium sexual states in the natural habitat (Chomnunti et al. 2012). Incumbomyces lentus has both MAT1-1 and MAT1-2, 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. The other carton species, I. delicatus only has a single MAT gene, with a structure similar with domatia species and A. fulminans. Among the known species of Chaetothyriales, only those isolated via their sexual state in nature (Capronia semimmersa, C. epimyces and C. coronata) have been proven to harbor two mating type genes, while all asexual species described to date have only a single mating type gene (Teixeira et al. 2017). Also the four domatia strains and Arthrocladium fulminans in Trichomieriacae harbored only a single MAT gene (Moreno et al. 2019). The MAT locus of the two species of Incumbomyces is remarkably different, with a large translocation in I. lentus where APN2, COX13 and APC5 are in the right flanking region. In I. delicatus they are in the left flanking region as in Arthrocladium and domatia fungi, but in different order. In general, the MAT location is extremely variable in Chaetothyriales (Teixeira et al. 2017) for reasons which have not been clarified.

Supplementary Information
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YQ and SH were major contributors in writing the manuscript. YQ, NS and BJF analyzed the data. VV, VM, YQK, and DMS modified and improved the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials
The datasets generated for this study can be found in the GenBank Accessions: JACJVT000000000 (I. delicatus) and JACJVT000000000 (I. lentus).

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
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Competing interests
The authors declare that they have no competing interests.

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