Adaptive evolution of sesquiterpene deoxyphomenone in mycoparasitism by *Hansfordia pulvinata* associated with horizontal gene transfer from *Aspergillus* species

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Abstract

Leaf mold caused by the ascomycete fungus *Cladosporium fulvum* is a devastating disease of tomato plants. The mycoparasitic fungus *Hansfordia pulvinata* is an effective biocontrol agent that parasitizes *C. fulvum* hyphae on leaves and secretes 13-deoxyphomenone, an eremophilane-type sesquiterpene, which was also identified as a sporulation-inducing factor in *Aspergillus oryzae*. Here, we identified deoxyphomenone biosynthesis (*DPH*) gene clusters conserved in both *H. pulvinata* and *Aspergillus* section *Flavi* including *A. oryzae* and *A. flavus*. Functional disruption of *DPH1* orthologous genes encoding sesquiterpene cyclase in *H. pulvinata*, *A. oryzae* and its close relative *A. flavus* revealed that deoxyphomenone in *H. pulvinata* had exogenic antifungal activity against the host fungus *C. fulvum* and controlled endogenic sporulation in *Aspergillus* species. Deoxyphomenone also inhibited mycelial growth of *C. fulvum* and the non-host tomato pathogen *Pseudocercospora fuligena*. Complete *DPH* clusters, highly similar to those in *H. pulvinata*, were exclusive to *Aspergillus* section *Flavi*, while species in other *Aspergillus* sections contained fragmented *DPH* clusters. A comparative genomics analysis revealed that these *DPH* gene clusters share a common origin and are horizontally transferred across large taxonomic distances from an ancestor of *Aspergillus* to *H. pulvinata*. Our results suggest that, after horizontal transfer, *H. pulvinata* maintained the *DPH* cluster as the inhibitory effect of deoxyphomenone on spore germination and mycelial growth contributed to its mycoparasitism on the host fungus *C. fulvum*.

Keywords: mycoparasite, deoxyphomenone, sporogen-AO 1, horizontal gene transfer, secondary metabolite, *Aspergillus flavus*, *Aspergillus oryzae*, *Cladosporium fulvum*, *Fulvia fulva, Hansfordia pulvinata, Dicyma pulvinata*

59 **INTRODUCTION**

60 The biotrophic filamentous fungus *Cladosporium fulvum* (Cooke) (syn. *Passalora fulva*,
61 *Fulvia fulva*), the causal agent of leaf mold of tomato, is an economic problem in most
62 countries that grow tomatoes (Videira et al., 2017; Mesarich et al., 2023). Following spore
63 germination, hyphae enter the leaf through stomata to absorb nutrients from its host in the
64 apoplast. After a few weeks, conidiophores emerge from the stomata and produce numerous
65 spores on the leaves, which appear as brown lesions. Colonization of tomato leaves by *C.*
66 *fulvum* is facilitated by numerous effector proteins that are secreted into the apoplast by the
67 fungus (de Wit, 2016). To combat *C. fulvum*, breeders have introduced *Cf* resistance genes,
68 which encode receptor-like proteins with extracytoplasmic leucine-rich repeats, into
69 commercial tomato cultivars from wild relatives (de Wit, 2016; Mesarich, 2023). Tomato *Cf*
70 gene encoded receptor-like proteins recognize corresponding effector proteins of *C. fulvum* as
71 virulence factors in the apoplastic region and trigger a hypersensitive response that rapidly
72 kills plant cells near the infection site, inhibiting the growth of the biotrophic pathogen and
73 preventing its spread (Stergiopoulos and de Wit, 2009). However, the occasional loss or
74 mutation of effector genes allows *C. fulvum* to escape the host immune system, resulting in
75 the emergence of new races (de Wit, 2016). Although single resistance genes have been
76 introduced into many tomato cultivars, overuse of resistant cultivars with only one or a few
77 *Cf* genes has led to the development of *C. fulvum* races that have overcome these resistance
78 genes. So far, 13 races have developed in Japan (Iida et al., 2015; Kubota et al., 2015;
79 Yoshida et al., 2021), and *C. fulvum* has evolved to sequentially overcome multiple *Cf* genes
80 (Rosa et al., 2023). The complex race structure of *C. fulvum* in Japan has enabled the fungus
81 to overcome all *Cf* genes present in commercial cultivars (Iida et al., 2015). A further
82 problem is that *C. fulvum* has developed resistance to several fungicides (Yan et al., 2007;
83 Watanabe et al., 2017), and applications of fungicides have to be reduced due to

84 environmental concerns. Thus, the use of antagonistic microorganisms instead of synthetic
85 fungicides is desired for sustainable control of leaf mold.
86 We serendipitously discovered white mycelia growing over lesions of leaf mold-infected
87 tomato leaves in a greenhouse and found that disease progression was inhibited on these
88 leaves (**Figure 1A**) (Iida et al., 2018). In further studies, we showed that the fungus
89 parasitizes hyphae of *C. fulvum* and inhibited all known races of this fungus (Iida et al.,
90 2018). The mycoparasitic fungus was identified as *Hansfordia pulvinata* (Berk. & M.A.
91 Curtis) S. Hughes [syn. *Dicyma pulvinata* (Berk. & M.A. Curtis) Arx] (Hughes, 1951;
92 Deighton, 1972; von Arx, 1981, 1982) based on its morphological characteristics and a
93 phylogenetic analysis (Iida et al., 2018). The fungus also has biocontrol activity against other
94 plant parasitic fungi, including *Mycosphaerella berkeleyi*, causing late leaf spot of peanut,
95 *Fusicladium heveae* causing South American leaf blight of rubber, and *Epichloë typhina*
96 causing choke disease of grasses (Peresse and Lepicard, 1980; Mitchell and Taber, 1986;
97 Mitchell et al., 1986; Mello et al., 2008; Alderman et al., 2010); it thus suppresses foliar
98 fungal diseases of different host plants. We also demonstrated that isolate 414-3 of *C. fulvum*
99 suppressed tomato leaf mold most effectively in the greenhouse (Iida et al., 2018). However,
100 the mechanism of this mycoparasitism of *H. pulvinata* against *C. fulvum* is not yet clear.
101 *H. pulvinata* produces 13-deoxyphomenone, an eremophilane-type sesquiterpene, as an
102 antifungal compound during vegetative growth (Tirilly et al., 1983). Around the same time,
103 this compound was also found and characterized (but named sporogen-AO 1) from
104 *Aspergillus oryzae*, which is used to produce koji that is used for the production of many
105 Japanese fermented foods such as Japanese sake and soy sauce (Tanaka et al., 1984a, 1984b).
106 This sesquiterpene is toxic to *C. fulvum*, which is parasitized by *H. pulvinata* but it induces
107 spore production in *A. oryzae*. In addition to its antifungal and sporogenic activities,
108 deoxyphomenone is also phytotoxic (Tansakul et al., 2014; Del Valle et al., 2015),

109 antimalarial (Daengrot et al., 2015), and cytotoxic to cancer cell lines (Tansakul et al., 2017;
110 Liu et al., 2021). However, its biosynthetic pathway in these fungi, the molecular basis of its
111 antifungal activity and spectrum, and the specificity its sporogenic activity in different
112 *Aspergillus* species have not been elucidated.

113 Fungal secondary metabolites play important roles in development, pathogenicity,
114 protection from biotic and abiotic stresses, antagonism, and communication with other
115 organisms (Keller, 2019). Because deoxyphomenone is produced by most strains of *H.*
116 *pulvinata* (Iida et al., 2018) and might be widely produced among *Aspergillus* species, it may
117 have mycoparasitic and/or ecological functions in these fungi. In this study, we investigated
118 the roles of deoxyphomenone in *H. pulvinata* and *Aspergillus* species. The antifungal
119 spectrum of the compound was determined for *C. fulvum* and other tomato fungal pathogens
120 that are not host fungi of *H. pulvinata*, such as *Pseudocercospora fuligena*, the causal agent
121 of tomato black leaf mold, which is phylogenetically closely related to *C. fulvum* and the
122 soilborne tomato pathogen *Fusarium oxysporum* f. sp. *lycopersici*. We identified
123 deoxyphomenone biosynthetic gene clusters in *H. pulvinata* and *Aspergillus* genome
124 sequences and functionally disrupted sesquiterpene cyclase genes to reveal the main function
125 of deoxyphomenone. Furthermore, we used a comparative genomics approach to elucidate
126 the origin of these gene clusters in *Aspergillus* and *H. pulvinata*.

127

128 **RESULTS**

129 **Antifungal activity of deoxyphomenone against plant pathogens**

130 *H. pulvinata* 414-3, the most effective biocontrol strain with a published draft genomic
131 sequence (Sushida et al. 2019) and the highest deoxyphomenone production among several
132 strains we tested, parasitizes *C. fulvum* hyphae in liquid culture without a carbon source (Iida
133 et al., 2018). In our present tests, *H. pulvinata* did not parasitize *C. fulvum* when grown on

134 nylon membranes on nutrient-rich potato dextrose agar (PDA). However, when the
135 membranes were transferred to nutrient-poor water agar, scanning electron micrographs
136 showed that hyphae of strain 414-3 penetrated the cell wall of *C. fulvum* (**Figure 1B**).
137 Although we previously reported that hyphae of *H. pulvinata* coil around the host hyphae, it
138 does not always do so, and it does not form appressorium-like structures, both common
139 attributes of the mycoparasitic *Trichoderma* fungi.
140 Since strain 414-3 produces about 100 μM deoxyphomenone in minimal medium (MM)
141 broth (Iida et al., 2018), we next evaluated its antifungal activity against *C. fulvum* using 120
142 μM commercial deoxyphomenone. At 120 μM, the compound inhibited colony formation
143 and hyphal elongation by more than 90% on MM agar (**Supplementary figure 1A, 1B**).
144 Deoxyphomenone also strongly inhibited spore germination at 120 μM, which was only 7.2%
145 after 24 h compared with 76.5% for the control spores treated with 1% (v/v) methanol
146 (**Figure 1C and Supplementary figure 1C**). When *C. fulvum* spores were treated with the
147 fungicide captan at 100 μM and after 24 h captan was replaced by distilled water, almost all
148 failed to germinate, but when deoxyphomenone (120 μM) was replaced by distilled water 24
149 h after treatment, about half of the treated spores did germinate (**Supplementary figure**
150 **S1C**). Spores treated continuously with deoxyphomenone or captan for 48 h failed to
151 germinate. Thus, the activity of deoxyphomenone is fungistatic; it inhibits the germination of
152 *C. fulvum* spores but does not kill them. Deoxyphomenone treatment did not affect the
153 structure of intracellular organelles or the cell membrane of the spores or induce necrosis in
154 tomato leaves even at concentrations as high as 80 μM, contrary to a previous report (Tirilly
155 et al., 1983) (**Supplementary figure S1D, E**). When plated on MM agar, external application
156 of deoxyphomenone also inhibited colony formation of plant pathogenic fungi that are not
157 hosts of *H. pulvinata*: the growth of *P. fuligena* phylogenetically closely related to *C. fulvum*,

158 was inhibited at the same concentrations as used for *C. fulvum*, but growth of *F. oxysporum* f.
159 sp. *lycopersici* was not affected (**Figure 1D**).
160
161 **Deoxyphomenone biosynthetic gene clusters are conserved between *H. pulvinata* and**
162 ***Aspergillus***
163 To identify the genes involved in deoxyphomenone biosynthesis in the genome sequences of
164 *H. pulvinata* and *A. oryzae*, we searched for homologous genes using the amino acid
165 sequence of aristolocene synthase Ari1 from *Penicillium roqueforti* (Proctor and Hohn,
166 1993), since the structure of deoxyphomenone has an aristolochene-like backbone (**Figure**
167 **1C**), and found two candidates (gene IDs, DIP_001954 and DIP_006271) in *H. pulvinata*
168 414-3 (Sushida et al. 2019) and one (GenBank accession XP_023093357) in *A. oryzae*
169 RIB40. Phylogenetic comparisons with other fungal sesquiterpene cyclases placed
170 DIP_001954 and XP_023093357 in the same clade as enzymes that catalyze the 1,10-
171 cyclization of farnesyl diphosphate, a common sesquiterpene precursor (**Supplementary**
172 **figure S2**). The deduced amino acid sequences of DIP_001954 and XP_023093357 had
173 61.6% similarity (49.1% identity) (**Supplementary figure S3**). We named DIP_001954
174 "*HpDPH1*" (deoxyphomenone biosynthesis gene 1) and the homologous gene
175 XP_023093357 "*AoDPH1*".
176 In the nucleotide sequences around *HpDPH1* we found genes encoding short-chain
177 dehydrogenase (*HpDPH2*), three cytochrome P450s (*HpDPH3, HpDPH4, HpDPH5*), and
178 major facilitator superfamily transporter (*HpDPH6*), altogether forming the deoxyphomenone
179 biosynthesis (*DPH*) gene cluster. We also found homologs of these genes in *A. oryzae*
180 (**Figure 2A**). Also genes encoding a lipase/thioesterase family protein and an integral
181 membrane protein were conserved in both fungi, but their function in deoxyphomenone
182 biosynthesis is unknown. The position and orientation of all genes were highly conserved, but

183 the loci of *AoDPH4* and *AoDPH5* were switched (**Supplementary figure S4**). Comparison
184 of these genes revealed that the number of exons in the pairs *HpDPH1/AoDPH1* and
185 *HpDPH5/AoDPH5* was identical and that the sequence similarity in each exon was conserved
186 among all pairs (**Supplementary figure S5**). We presumed that the deoxyphomenone
187 biosynthetic pathway involved cyclization of farnesyl diphosphate by HpDph1, followed by
188 oxygenation and epoxidation by cytochrome P450s HpDph3, HpDph4 and HpDph5, and
189 subsequently hydroxylated by HpDph2 (**Figure 2B**). The putative substrate binding pocket
190 that is conserved in the major facilitator superfamily transporter (Quistgaard et al., 2016) was
191 also conserved in HpDph6/AoDph6 (**Supplementary figure S6**).
192 To obtain a comprehensive view of the distribution of the *DPH* cluster in other fungi, we
193 extended the search to *HpDPH1* homologues in the MycoCosm database
194 (mycocosm.jgi.doe.gov; Grigoriev, 2014). Genes partially homologous to *HpDPH1* were
195 found in 139 fungal genomes of the *Eurotiomycetes*, *Sordariomycetes* and *Dothideomycetes*
196 (**Supplementary table S1**). Besides the *DPH* gene cluster present in *H. pulvinata*, other *DPH*
197 genes were detected as a gene cluster in *Aspergillus* species only (*Eurotiomycetes*), but not in
198 the *Xylariales* (*Sordariomycetes*), to which *H. pulvinata* belongs, which suggests that the
199 latter fungus has obtained this cluster by horizontal transfer. Notably, the cluster was
200 completely conserved in *Aspergillus* species section *Flavi* to which *A. oryzae* belongs
201 (**Supplementary figure S7 and Figure 2A**).
202 We then analyzed expression of the putative deoxyphomenone biosynthetic genes during
203 mycoparasitism of *H. pulvinata* 414-3 *in vitro* using quantitative real-time PCR. *H. pulvinata*
204 never parasitize *C. fulvum* on the nutrient-rich PDA, but it did so on the nutrient-poor water
205 agar (**Figure 2A**). The expression of the *HpDPH* genes in *H. pulvinata* did not differ when
206 grown on PDA medium or on water agar (**Figure 3**). In contrast, the expression of *HpDPH*
207 genes was significantly upregulated when *H. pulvinata* parasitized *C. fulvum* on water agar.

208 Expression of *HpDPH1*, *HpDPH2* and *HpDPH3* genes, which encode sesquiterpene cyclase,
209 short-chain dehydrogenase, and cytochrome P450, respectively, were significantly induced
210 during mycoparasitism (**Figure 3**). Expression of DIP_001952 (lipase/thioesterase family
211 protein) and DIP_001953 (integral membrane protein) genes also increased, suggesting that
212 these proteins are associated with deoxyphomenone biosynthesis or mycoparasitism.
213

214 **Deoxyphomenone is the main antifungal compound of *H. pulvinata* effective against *C.***
215 ***fulvum***

216 Since the *DPH* gene cluster was also found in the genus *Aspergillus*, we assayed for the
217 presence of deoxyphomenone in the MM culture filtrate from 22 strains of *A. oryzae* and 12
218 strains of *A. flavus*. In contrast to *H. pulvinata* 414-3, which produced deoxyphomenone as
219 high as about 80 μM, *A. oryzae* and *A. flavus* strains produced only low levels of
220 deoxyphomenone: the highest concentrations in *A. oryzae* and *A. flavus* strains are about 0.8
221 μM and about 3.5 μM, respectively (**Supplementary figure S8**). We then measured
222 deoxyphomenone by the most productive strains of *A. oryzae* (RIB40) and *A. flavus*
223 (NBRC114564) in different culturing conditions and found that both strains produced the
224 compound when grown as still cultures in MM broth (**Supplementary figure S9**).
225 Interestingly, neither strain produced deoxyphomenone at 35 °C, the optimum temperature
226 for vegetative growth and sporulation, but showed maximal production at 25 °C, at which
227 vegetative growth and sporulation are retarded. Similarly, *H. pulvinata* showed the highest
228 production of deoxyphomenone at 28 °C, a temperature at which vegetative growth is also
229 retarded (**Supplementary figure S9**).

230 To determine the function of *HpDPH1* in deoxyphomenone biosynthesis in *H. pulvinata*
231 414-3, we generated Δ*HpDPH1* knockout mutant strains of 414-3 by homologous
232 recombination through *Agrobacterium tumefaciens*-mediated transformation

233 (**Supplementary figure S10**). Quantitative analysis by LC-MS/MS revealed the presence of
234 deoxyphomenone in the culture filtrate of wild-type 414-3 strain, but none in that of the
235 *ΔHpDPH1* mutant strain KO10 (**Figure 4A**). Strain 414-3 started to produce
236 deoxyphomenone at 3 d and increased until 15 d, whereas strain KO10 did not produce any
237 (**Figure 4B**). The culture filtrate of 14-day-old cultures of 414-3 containing deoxyphomenone
238 strongly inhibited the germination of *C. fulvum* spores, whereas those of the Δ*HpDPH1*
239 mutant strains KO10 and KO37 showed no inhibitory activity, suggesting that
240 deoxyphomenone is the main compound contributing to the antifungal activity of *H.*
241 *pulvinata* (**Figure 4C**). The antifungal activity was partially restored in the loss-of-function
242 Δ*HpDPH1* mutant strain (CO10A) after introduction of a functional *AoDPH1* gene (**Figure**
243 **4C**). This is most likely explained by restoration of deoxyphomenone production that
244 accumulated in the culture filtrate of CO10A. The Δ*HpDPH1* mutant KO10 parasitized *C.*
245 *fulvum* similar to the wild-type 414-3 strain, with no differences in spore number or
246 vegetative growth, as we expected previously (Iida et al., 2018) (**Supplementary figure**
247 **S11**).
248
249 **The function of deoxyphomenone differs between *H. pulvinata* and the *Aspergillus***
250 **species**
251 We also generated knockout strains of the homologous gene *AfDPH1* in *A. flavus*
252 NBRC114564, which produced more deoxyhomenone than *A. oryze* RIB40 (**Supplementary**
253 **figure S8**). LC-MS/MS analysis showed that wild-type strain and ectopic transformants
254 produced deoxyphomenone, while the Δ*AfDPH1* mutant strains produced none (**Figure 5A**).
255 As mentioned earlier, deoxyphomenone (sporogen-AO 1) was initially identified as a
256 sporogenic factor in *A. oryzae* (Tanaka et al., 1984b, 1984a). Three Δ*AfDPH1* mutant strains
257 grown on MM agar produced significantly less spores than the wild-type NBRC114564

258 (**Figure 5B, C**). Two ectopic transformants produced similar quantities of spores as the wild-
259 type. Conversely, a Δ*AoDPH1* mutant of *A. oryzae* RIB40 completely lost the ability to
260 produce deoxyphomenone, but its spore production and colony morphology were similar to
261 those of the wild-type and ectopic transformants (**Supplementary figure S12**).
262 We then studied the phenotype of the fungal strains after exposure to deoxyphomenone
263 on MM. Since *H. pulvinata* 414-3 yielded about 30–100 μM deoxyphomenone during
264 vegetative growth, we confirmed that deoxyphomenone was fungistatic against *C. fulvum*
265 over a wide range of concentrations; inhibition by deoxyphomenone was concentration-
266 dependent; inhibition of spore germination of *C. fulvum* was around 25% at 10 μM, reaching
267 about 90% at 80 μM (**Figure 6A**). Thus, the concentration of deoxyphomenone secreted by
268 strain 414-3 seems sufficient to inhibit *C. fulvum* spore germination. As Tanaka et al. (1984b)
269 treated *A. oryzae* strains with higher concentrations of deoxyphomenone (sporogen-AO 1) to
270 test sporogenic activity, we also tested concentrations up to 120 μM against *A. oryzae* RIB40
271 and *A. flavus* NBRC114564. In *Aspergillus*, deoxyphomenone treatment also affected
272 sporulation in a concentration-dependent manner, but the effect varied among different
273 species: compared to no treatment, treatment with 120 μM deoxyphomenone reduced spore
274 production by RIB40, but increased it in NBRC114564 by ~90% (**Figure 6B**). While
275 deoxyphomenone did not affect colony growth or sporulation of *H. pulvinata* strain 414-3,
276 colony morphology and formation of aerial hyphae were altered in *Aspergillus* strains, and
277 colony growth was also slightly inhibited in RIB40, indicating that RIB40 is less tolerant to
278 this sesquiterpene than 414-3 (**Figure 6B and Supplementary figure S13**). These results
279 show that deoxyphomenone has quite different activities in different fungi; it has exogenic
280 antifungal activity against the host fungus and affects endogenic sporogenesis in *Aspergillus*
281 species.
282

283 **Deoxyphomenone biosynthetic gene cluster was horizontally transferred from an**
284 ***Aspergillus* ancestor to *H. pulvinata***
285 Next, we analyzed the commonality and conservation of the *DPH* clusters in genomic
286 sequences of *Aspergillus* species. Complete *DPH* clusters were detected only in *Aspergillus*
287 species from the section *Flavi* (Group I, **Figure 2A**), and the protein sequences of HpDph2 to
288 HpDph6 in these species were highly conserved, with ~70% pairwise similarity to those of *H.*
289 *pulvinata* (**Figure 7**). Meanwhile, partial *DPH* clusters were present in *Aspergillus* species of
290 the section *Circumdati* (Group II: *HpDPH1*, *HpDPH2*, *HpDPH3*) and the sections *Nidulantes*
291 and *Clavati* (Group III: *HpDPH1*, *HpDPH2*, *HpDPH4*) (**Figure 7**). Group II species also
292 contained a lipase/thioesterase family protein- and an integral membrane protein-encoding
293 genes downstream of *HpDPH1*, the same as in Group I (**Figure 7 and Supplementary**
294 **figure S7**). Group III further lacked the commonality and conservation of the *DPH* clusters:
295 the similarities with *HpDPH1* and *HpDPH2* genes were much lower than in the other two
296 groups, the position and the order of *DPH2* and *DPH4* genes in the genome sequence were
297 quite different, and unrelated genes were interspersed in the cluster (**Figure 7** and
298 **Supplementary figure S7**). These results suggested that Group II and III species lost parts of
299 the *DPH* cluster and no longer produced deoxyphomenone.
300 To infer the evolutionary origin of the *DPH* clusters in *H. pulvinata* and *Aspergillus*, we
301 analyzed the evolutionary divergence of the clusters among 60 fungal genomes using
302 genomic and phylogenetic approaches with the NOTUNG algorithm, which reconciles
303 differences between species trees and gene trees by inferring gene duplications, transfers, and
304 losses (Stolzer et al., 2012). Reconciliation of the species tree with the HpDph1 protein tree
305 detected a transfer event (T1 in **Figure 8**) early in *Aspergillus* section *Circumdati*, indicating
306 a transfer of *HpDPH1* of *H. pulvinata* from Group II (*Circumdati*) (**Supplementary figure**
307 **S14**). Likewise, the HpDph2 and HpDph4 protein trees indicated transfers from Group I

308 (section *Flavi*) to *H. pulvinata* (T2 in **Figure 8**). Since the *DPH* clusters are highly conserved
309 in *H. pulvinata* and *Aspergillus*, these results suggest an evolutionary scenario in which the
310 *DPH* cluster was transferred from the common ancestor of Groups I and II (sections *Flavi*
311 and *Circumdati*) to *H. pulvinata* at the same time and that genes were lost in time in
312 *Aspergillus* species (**Figure 8**). Since all duplication events detected in *DPH* genes occurred
313 after the horizontal transfer events (**Supplementary figure S14**), it is unlikely that the
314 duplication is associated with the transfer of the *DPH* cluster. Altogether these results suggest
315 that the *DPH* cluster arose before speciation within the *Aspergillus* genus. Finally, because
316 we inferred the horizontal transfer of the *DPH* cluster from *Aspergillus* to *H. pulvinata*, we
317 investigated the association of deoxyphomenone with the mycoparasitism. Contrary to our
318 expectations, *H. pulvinata* 414-3 did not directly penetrate the hyphae of *A. oryzae* RIB40, as
319 observed with the host fungus *C. fulvum*, but physical contact such as adhesion to or coiling
320 around the hyphae of the RIB40 was detected (**Supplementary figure S15**).
321

322 **DISCUSSION**

323 Strict regulation of expression through the physical linkage of genes involved in the same
324 biosynthetic pathway leads to physiological economization; thus, biosynthetic genes for
325 secondary metabolites in fungi are often maintained in clusters (Hagee et al., 2020), which
326 allows for the rapid conversion of potentially toxic or chemically unstable intermediate
327 products (McGary et al., 2013). In the present study, the *DPH* clusters were highly conserved
328 between *H. pulvinata* and *Aspergillus* section *Flavi*, suggesting that the final product,
329 deoxyphomenone, plays critical roles in distinct stages of the life cycle of these fungi. Indeed,
330 it acts as an antifungal agent of the mycoparasite *H. pulvinata* against the host fungus *C.*
331 *fulvum* and controls sporulation at least in *A. flavus*. The correlation of these fungal clusters
332 can be explained through the most parsimonious evolutionary scenario, which is constructed

333 by reconciling the species tree with the *DPH* protein tree in consideration of the costs of
334 evolutionary events (i.e., gene duplications, transfers, and losses) (Stolzer et al., 2012).
335 Interestingly, we inferred that the *DPH* cluster was horizontally transferred across a large
336 taxonomic distance from the genus *Aspergillus* (*Eurotiomycetes*) to *H. pulvinata*
337 (*Sordariomycetes*), suggesting that *H. pulvinata* maintained the cluster as deoxyphomenone
338 production seemed to be more conductive to mycoparasitism. Horizontal gene transfer is not
339 rare in fungi (Mehrabi et al. 2011; Rokas et al., 2018); gene clusters for the biosynthesis of
340 products such as fumonisin (Proctor et al., 2013, Khaldi and Wolfe, 2011), host-specific
341 toxins in *Alternaria* species (Tsuge et al., 2016), and cercosporin (de Jonge et al., 2018) have
342 been horizontally transferred across classes, but the final products function in the recipient
343 fungi basically in a similar way.
344 We deduced the deoxyphomenone biosynthetic pathway from the component genes of
345 the *DPH* cluster in *H. pulvinata* and *Aspergillus*. The bicyclic eremophilane-type
346 sesquiterpene deoxyphomenone is probably formed by the cyclization of farnesyl
347 pyrophosphate catalyzed by sesquiterpene cyclase (Proctor and Hohn, 1993), followed by
348 oxygenation and epoxidation of the double bond in *H. pulvinata* and *Aspergillus*. Although
349 deoxyphomenone has also been detected in *Penicillium* species, which are taxonomically
350 close to *Aspergillus*, the *DPH* cluster was not detected in the published genome sequences of
351 *Penicillium* species; thus, the pathway of deoxyphomenone biosynthesis is likely different
352 between the two genera. On the basis of a relaxed molecular clock analysis, *Aspergillus* and
353 *Penicillium* likely diverged 94.0 million years ago (mya) with *Aspergillus* sections *Flavi* and
354 *Circumdati* originating 51.1 mya (Barelli et al., 2015; Steenwyk et al., 2019). Because the
355 *DPH* clusters are fully or partially conserved in *Aspergillus* sections *Flavi* and *Circumdati,*
356 we presume that their horizontal transfer to *H. pulvinata* occurred at least before the common
357 ancestor of these sections.

358 It is still unclear how *H. pulvinata* acquired the *DPH* clusters from the ancestral
359 *Aspergillus*. Since horizontal transfer of foreign DNA first requires contact between fungal
360 cells, the involvement of anastomosis between conidia, germ tubes or hyphae, and subsequent
361 heterokaryon formation, have been proposed (Kubicek et al., 2019; Fleißner et al., 2022;
362 Bucknell and McDonald, 2023). *H. pulvinata* has a wide host range, including plant
363 pathogens (Peresse and Lepicard, 1980; Mitchell and Taber, 1986; Mitchell et al., 1986;
364 Mitchell et al., 1987; Mello et al., 2008; Alderman et al., 2010), and plant pathogens in
365 *Aspergillus* sections *Flavi* and *Circumdati* may provide opportunities for contact with *H.*
366 *pulvinata* on plants and subsequent gene transfer. Given the conservation of *DPH* clusters
367 revealed in this study and the nature of the recipient fungus, we propose to add
368 mycoparasitism as one of the mechanisms that facilitated horizontal gene transfer between
369 fungi. Mycoparasitic *Trichoderma* species have been suggested to have acquired by
370 horizontal transfer nearly half its genes encoding plant cell wall-degrading carbohydrate-
371 active enzymes and auxiliary proteins enabling them to be parasitism on a broad range of
372 *Ascomycota* (Druzhinina et al., 2018). *H. pulvinata* 414-3 did not establish a direct parasitic
373 relationship with *A. oryzae* RIB40 as it did with *C. fulvum*, and no distinct anastomosis was
374 observed, but it firmly adhered to and coiled around RIB40 hyphae, indicating basic
375 compatibility facilitating horizontal between the two fungi. Since *H. pulvinata* completely
376 kills the host *C. fulvum*, *C. fulvum* might not be a stable source of foreign DNA, but rather *H.*
377 *pulvinata* might have acquired the gene cluster in direct contact with nonhost fungi. In the
378 genomic sequence of *H. pulvinata*, a gene cluster derived from *Aspergillus* was found in this
379 study, but no horizontal gene transfer between host fungi, including *C. fulvum*, has been
380 found so far. Although transduction by mycoviruses or propagation of retrotransposons could
381 also have facilitated horizontal gene transfer (Richards et al., 2006), no transposon-like
382 sequences were detected around the *DPH* cluster.

383 Several environmental cues such as temperature, light, nutrients, pH, and competing or
384 synergistic organisms influence fungal activity, including transcriptional regulation of
385 secondary metabolism–associated gene clusters (Keller, 2019). Aflatoxin production in *A.*
386 *flavus* is generally upregulated at a suboptimal 30 °C (Ching'anda et al., 2021). Similarly, we
387 found that deoxyphomenone production was also highest at temperatures unsuitable for
388 growth of *H. pulvinata* and *Aspergillus* species (**Supplementary figure S8**). In *A. flavus*,
389 Δ*AfDPH1* mutants that no longer produced deoxyphomenone formed significantly less
390 spores. These results suggest that in at least *A. flavus*, deoxyphomenone stimulates
391 sporulation in poor nutritional environments. In addition, *A. flavus* strains tended to produce
392 more deoxyphomenone than *A. oryzae* strains, but the level was not sufficiently high for
393 antifungal activity. Gibbons et al. (2012) reported that the expression levels of many
394 secondary metabolite gene clusters of *A. oryzae*, including those for *DPH* and aflatoxin, were
395 repressed compared with those of *A. flavus*. *A. oryzae*, used to ferment rice to produce
396 Japanese sake, is probably derived from *A. flavus*, a pathogen toxic to plants and animals
397 (Gibbons et al., 2012). *A. oryzae* is cultured with the budding yeast *Saccharomyces cerevisiae*
398 during sake brewing and might have lost the ability to produce aflatoxin during its
399 domestication from *A. flavus* (Watarai et al., 2019) because aflatoxin is genotoxic to yeast
400 (Keller-Seitz et al., 2004). Since *A. oryzae* is usually grown under optimal fermentation
401 conditions, the gene encoding deoxyphomenone required for sporulation by *A. flavus* in
402 unfavorable conditions may also have been silenced or lost in *A. oryzae*. In fact, external
403 application of deoxyphomenone inhibited sporulation and colony formation only in *A.*
404 *oryzae*, indicating that *A. oryzae*, which produces only low levels of the compound, is less
405 tolerant to this sesquiterpene (**Supplementary figure S13**). The mycoparasite *H. pulvinata*
406 also produced deoxyphomenone at temperatures unfavorable for its growth, but in the
407 presence of its host fungus *C. fulvum*, expression of the *HpDPH* genes was strongly induced,

408 suggesting that deoxyphomenone might be crucial for mycoparasitic activity of *H. pulvinata*.
409 However, the *ΔHpDPH1* mutant strains of *H. pulvinata* parasitized *C. fulvum*, comparable to
410 the wild-type strain, supporting that deoxyphomenone is not required directly for its
411 mycoparasitism (Iida et al., 2018). Spore germination of *C. fulvum* treated with
412 deoxyhomenone for 24 h was halved, also suggesting an epidemiological effect on the
413 mycoparasitism of *H. pulvinata*.
414 Deoxyphomenone did not affect the organelles in *C. fulvum* spores, and the specific
415 target is still unknown. Although it is not directly involved in the mycoparasitism of *H.*
416 *pulvinata* and is not toxic to plants, it had significant fungistatic activity against plant
417 pathogens—the host *C. fulvum* and the nonhost *P. fuligena* of *H. pulvinata*—which might
418 indicate a potential effect on biotrophic fungal pathogens of tomato plants. *H. pulvinata*
419 remains an efficient biocontrol agent that can be used as a biofungicide to suppress races and
420 fungicide-tolerant strains of *C. fulvum* that cause problems in tomato crops world-wide (Iida
421 et al., 2015; Mesarich et al., 2023). Among *Aspergillus* species commonly used in
422 fermentation, the use of deoxyphomenone to control sporulation should promote high
423 efficiency for industrial fermentation and enzyme production.
424

425 **MATERIALS AND METHODS**

426 **Growth conditions for fungal strains and plants**

427 Strains of *H. pulvinata* 414-3 and *C. fulvum* CF301 were grown on PDA (half-strength, BD
428 Difco, Franklin Lakes, NJ, USA), MM agar or broth (15 g sucrose, 5 g ammonium tartrate, 1
429 g NH_4NO_3, 1 g KH_2PO_4, 0.5 g $MgSO_4 \cdot 7H_2O$, 0.1 g NaCl, 0.1 g $CaCl_2 \cdot H_2O$, 25 μL 0.2 mg/mL
430 biotin, 15 g agar and 1 mL trace elements per liter) at 25 °C in the dark for 1 and 2 weeks,
431 respectively, then spores were collected in sterile distilled water. *A. oryzae* and *A. flavus*
432 strains (**Supplementary figure S7**) from NITE-NBRC (www.nite.go.jp/nbrc/catalogue/)

433 were cultured on PDA, MM agar and broth, and Czapek-Dox agar and broth (3 g $NaNO_3$, 2 g
434 KCl, 1 g KH_2PO_4, 0.5 g $MgSO_4 \cdot 7H_2O$ 20 g glucose and 15 g agar per liter adjusted to pH 6.5)
435 at 30 °C in the dark for a week, and spores were collected in sterile distilled water containing
436 1% (v/v) Tween 20. The concentration of spores was determined using a hemocytometer.
437 Strains were maintained on PDA at 25 °C for a few months. Spore suspensions containing
438 10% v/v glycerol of all fungi except *H. pulvinata* were preserved at –80 °C for long-term
439 storage; *H. pulvinata* was stored in 10% v/v glycerol with 5% w/v trehalose.
440 For examining mycoparasitic interactions on tomato leaves, tomato cultivar
441 Moneymaker, which lacks any apparent *Cf* resistance gene, was grown in plastic pots for 3
442 weeks in a climate chamber at 25 °C with 16 h light/8 h dark. Seedlings were transplanted to
443 soil in the greenhouse and grown for 3 weeks. The lower sides of tomato leaves were sprayed
444 with a spore suspension of *C. fulvum* CF301 (1×10^5 spores/mL). After 2 weeks, a spore
445 suspension of *H. pulvinata* 414-3 (1×10^5 spores/mL) was sprayed on the brown lesions that
446 had formed on the abaxial surfaces of the leaves. White mycelial patches of *H. pulvinata*
447 were observed a week after inoculation.
448
449 **Electron microscopy**
450 *C. fulvum* CF301 or *A. oryzae* RIB40 were cultured on a nylon membrane (0.45 μm;
451 GVS Japan, Tokyo, Japan) placed on PDA in a petri dish and incubated as described above.
452 The membranes were then transferred to water agar and incubated for a week in the same
453 conditions. A spore suspension of *H. pulvinata* (1×10^5 spores/mL) was sprayed on the test
454 colony on the membrane, then white colonies were observed 1 week later.
455 Small pieces (1×1 cm^2) were cut from the membrane and fixed in 2.5% (v/v)
456 glutaraldehyde in 100 mM cacodylate buffer (pH 7.4) overnight at 4 °C, then washed three
457 times in the buffer, fixed in 1% (w/v) osmium tetroxide in the buffer for 1 h at room

458 temperature and washed three times in distilled water. Specimens were then dehydrated
459 through a graded ethanol series, critical-point dried in absolute ethanol using Leica EM
460 CPD300 critical point dryer (Leica Microsystems, Wetzlar, Germany), then mounted on
461 stubs, coated with platinum-palladium, and observed with a Hitachi SU-8220 scanning
462 electron microscope (Tokyo, Japan).

463 For observing the ultrastructure of *C. fulvum* spores with transmission electron
464 microscopy (TEM), spores of strain CF301 were treated with deoxyphomenone (120 μM) at
465 25 °C for 24 h in the dark. Spores were collected by centrifugation, washed three times with
466 sterile water, then fixed in phosphate-buffered 2% glutaraldehyde at 4 °C overnight. The
467 samples were again collected by centrifugation, then treated with 2% potassium
468 permanganate in phosphate buffer for 1 h at room temperature and post-fixed in 2% w/v
469 osmium tetroxide in phosphate buffer for 2 h at 4 °C. The samples were dehydrated in a
470 graded ethanol series and embedded in epoxy resin (Fujifilm Wako, Osaka, Japan) at 60 °C
471 for 48 hours. Ultrathin sections were cut using an ultramicrotome Leica UCT Ultracut (Leica
472 Microsystems), then stained with uranyl acetate for 15 min and lead solution for 5 min and
473 observed with a Hitachi TEM H-7600.

474

475 **Assays of antifungal activity and plant toxicity of deoxyphomenone**

476 *C. fulvum* spores were treated with deoxyphomenone [(+)-13-deoxyphomenone, sporogen-
477 AO1; Apollo Scientific, Bredbury, UK] in 1% methanol (v/v) at 25 °C in the dark for 24 h.
478 Then samples were observed for germination and hyphal elongation using a Nikon E600 light
479 microscope (Tokyo, Japan).

480 The antifungal activity of deoxyphomenone was compared with that of an *N*-halo-
481 alkylthioimide fungicide, captan (orthocide80; Arysta Life Science, Tokyo, Japan). *C. fulvum*
482 spores (1×10^3 cells/mL) were treated with distilled water, deoxyphomenone (120 μM), or

483 captan (100 μM) on a hydrophobic TF0808 glass slide (Matsunami Glass, Osaka, Japan) for
484 24 h at 100% humidity, then each solution was replaced with fresh solution, and samples
485 incubated another 24 h. Germinated spores were then counted using the Nikon light
486 microscope. For the controls, dH_2O or 1% methanol (v/v) were used. Each treatment was
487 done in triplicate, and at least 200 spores and hypha were assessed for each treatment.
488 Spore suspensions of the strains *C. fulvum* CF301, *P. fuligena* Pf17923, kindly
489 provided by K. Nakajima and T. Kawakami, and *F. oxysporum* f. sp. *lycopersici* CK3-1 were
490 adjusted to 1×10^6 spores/mL and 10 μL were dropped on MM agar supplemented with
491 different concentrations of deoxyphomenone (20 to 120 μM). The strains were cultured at
492 25 °C in the dark for 2 weeks or 3 days.
493 To evaluate the toxicity of deoxyphomenone on tomato plants, we injected the abaxial
494 surface of three leaves on each of two 1-month-old tomato plants with about 20 μL of
495 deoxyphomenone (20 to 80 μM) or with 1% methanol (v/v) as a control. The plants were then
496 incubated in a climate chamber for 1 week, then leaves were examined for necrosis.
497
498 **Phylogenetic analyses of deoxyphomenone biosynthetic gene clusters**
499 Deoxyphomenone biosynthetic genes and *DPH* clusters were initially identified in the
500 genomic sequences of *H. pulvinata* 414-3 (Sushida et al., 2019) and *A. oryzae* RIB40
501 (Machida et al., 2005) using the BLAST program (blast.ncbi.nlm.nih.gov). Sequences shown
502 in **Supplementary table S1 and S2** were aligned using MAFFT online version 7 (Katoh and
503 Standley, 2013) with default parameters and trimmed using TrimAl version 1.4 (Capella-
504 Gutiérrez et al., 2009). Maximum likelihood phylogenetic trees were constructed using
505 RAxML version 8.2.12 with 1000 bootstraps; the PROTCATAUTO and GTRCAT models
506 were selected for amino acid and nucleotide sequences, respectively (Stamatakis, 2014). Dot
507 plot alignments were generated from the MAFFT analysis (**Supplementary figure S4**).

508 Pairwise sequence alignments of *DPH6* homologous genes were performed using EMBOSS
509 Needle (Madeira et al. 2022).
510 The phylogenetic species tree in **Figure 8** was constructed using maximum likelihood
511 based on the 500 monocore genes (a single homolog in each of the species) using CVtree
512 version 3.0 (k = 16) (Qi et al., 2004) by uploading the genomic information for the 60
513 published fungi shown in **Supplementary table S1**.
514 Reconciliation analysis between species and gene trees was performed using NOTUNG
515 v.2.9 (Stolzer et al., 2012) according to the instructions (version 2.8 beta) to infer the
516 evolutionary trajectories of the *DPH* clusters. Event scores were calculated as the total costs
517 of duplications, transfers and losses. Costs/weights were set as duplications (D), 1.5; transfers
518 (T), 8.0; losses (L), 1.0 (ratio D:T:L is 1:5.3:0.67) for amino acid sequences, and D, 1.5; T,
519 6.0; L, 1.0 (ratio D:T:L is 1:4:0.67) for nucleotide sequences for the gene clusters.
520
521 **Quantitative detection of deoxyphomenone**
522 *H. pulvinata* (1×10^6 spores) was cultured in 50 mL of MM broth with shaking at 25 °C in a
523 light/dark cycle of 16 h/8 h for 2 weeks. *A. oryzae* and *A. flavus* (1×10^6 spores) were
524 incubated in MM broth without shaking at 25 °C in the dark for 2 weeks. The culture
525 supernatants were collected by centrifugation and filtered through a 0.45 μm filter (Merk,
526 Darmstadt, Germany). Deoxyphomenone was quantified using a 4000 QTRAP LC-MS/MS
527 system (Sciex, MA, US) equipped with a 1290 series HPLC system (Agilent Technologies,
528 CA, US) as previously reported (Iida et al., 2018). Commercial deoxyphomenone (Apollo
529 Scientific) was used as a standard.
530
531 **Construction of transformation vectors**
532 Genomic DNA was extracted from *H. pulvinata* and *A. oryzae* mycelia grown in MM broth

533 for a week using Nucleo-Mag Plant (TakaraBio, Shiga, Japan) according to the
534 manufacturer's instructions. The DNA was then amplified by PCR using PrimeSTAR GXL
535 Premix (TakaraBio) or KOD ONE (TOYOBO, Osaka, Japan) according to the instructions.
536 For generating the gene knockout vector, the upstream (1.2 kb) and downstream (1.0 kb)
537 regions of *HpDPH1* were amplified from *H. pulvinata* genomic DNA using primer sets
538 Dpprx2LF1/Dpprx2LR1 and Dpprx2UF1/Dpprx2UR1, respectively (**Supplementary table**
539 **S3**). Geneticin-resistance gene (*gen*) cassettes were amplified from pRM254 (Mehrabi et al.,
540 2015) using primers pRM254 attL1_F/pRM254 attL1_R. These primers contain a 5′
541 overhang sequence to overlap the sequences at the ends of the linearized plasmid or the *gen*
542 cassette (**Supplementary table S3**). Plasmid pPM43GW was amplified using the primers
543 pPM43GW_RB_F and pPM43GW_RB_R to linearize and remove the *ccdB* gene, which
544 encodes a product that is lethal to bacterial cells. Four fragments were combined to generate
545 the gene replacement vector pGW43_dpprx2ko using the In-Fusion EcoDry Cloning Kit
546 (TakaraBio).
547 To introduce the *AoDPH1* gene into Δ*HpDPH1* strain KO10, *AoDPH1* (without introns)
548 and hygromycin resistance gene (*hph*) cassettes were synthesized by VectorBuilder, Inc.
549 (Chicago, IL, USA). The *AoDPH1* and *hph* genes were driven under the promoters of the *A.*
550 *nidulans trpC* gene and *Cochliobolus heterostrophus gpd1* (NCBI accession X02390.1 and
551 X63516.1), respectively (**Supplementary figure S9A**). Synthesized DNA was inserted into
552 the blunt end of the PvuII-digested plasmid pPZP-PvuII kindly provided by Prof. Chihiro
553 Tanaka.
554 To construct gene replacement vector for *AoDPH1* and *AfDPH1* gene, a plasmid pSH75
555 (Kimura and Tsuge 1993) was digested with *Bgl* II and *Hin*d III, and the resulting 2.4 kb
556 fragment was used as a backbone. Marker gene cassettes (4.7 kb) containing a pyrithiamine
557 resistant gene (*ptrA*) and an *EGFP* gene cassette were amplified from a plasmid

558 pPTREXeGFP using the primers pPTREXeGFP_F and pPTREXeGFP_R. The upstream (1.5
559 kb) and downstream (1.5 kb) regions of *AoDPH1* were amplified from genomic DNA of *A.*
560 *oryzae* RIB40 using primer sets AoDPH1_LB_F/AoDPH1_LB_R and
561 AoDPH1_RB_F/AoDPH1_RB_R, respectively (**Supplemental table S3**). These primers
562 contain a 5′ overhang sequence to overlap the sequences at the ends of the pSH75 fragment
563 or the marker cassette (**Supplementary table S3**). These four fragments were combined
564 using the In-Fusion EcoDry Cloning Kit (TakaraBio), then the resulting plasmid was named
565 pSH75PTRdAoDPH1. The plasmid was linearized by digestion with *Swa* I and used for
566 fungal transformation.
567 Plasmid vectors were introduced into *Escherichia coli* DH5α (NIPPON GENE, Tokyo,
568 Japan) and extracted using MagExtractor -Plasmid- (TOYOBO) according to the instructions.
569 The correct orientation of the fragments in the final constructs was confirmed by PCR.
570
571 **Fungal transformation**
572 All transformations were done as previously described (Okmen et al., 2013) with some
573 modifications for *H. pulvinata*. Briefly, *A. tumefaciens* (*Rhizobium radiobacter*) strain AGL-
574 1 was transformed with the plasmid vectors used for the *H. pulvinata* transformation using
575 the Gene Pulser electroporator (Bio-Rad Laboratories, Hercules, CA, USA) according to the
576 instructions, then grown on LBman agar (10 g tryptone, 10 g NaCl, 5 g yeast extract, 10 g
577 mannitol, 20 g agar per liter) supplemented with appropriate antibiotic at 28 °C for 3 days.
578 Colonies were then collected and resuspended in IM broth (1 g glucose, 2.05 g K_2HPO_4, 1.45
579 g KH_2PO_4, 0.15 g NaCl, 0.50 g $MgSO_4 \cdot 7H_2O$, 0.07 g $CaCl_2 \cdot 2H_2O$, 0.5 g $(NH_4)_2SO_4$, 0.5%
580 (w/v) glycerol, 8.53 g 2-(*N*-morpholino)ethanesulfonic acid [pH 5.3] per liter) supplemented
581 with 200 μM acetosyringone at an optical density at 600 nm (OD_{600}) of 0.2. The bacterial
582 suspension (200 μL) was mixed with 200 μL of a spore suspension of *H. pulvinata* (1.0×10^5

583 cells/mL), then the suspension was spread on a nylon membrane (GVS Japan) that had been
584 placed on IM agar (IM; 20 g per liter) in a plate. After 2 days of incubation in the dark at
585 25 °C, the membranes were transferred to PDA supplemented with either 200 μg/mL
586 geneticin (G418 sulfate; Fujifilm Wako) or 100 μg/mL hygromycin (Fujifilm Wako), and 50
587 μg/mL meropenem trihydrate (Fujifilm Wako). Transformed fungal colonies appeared after a
588 week were transferred to new PDA plates supplemented with geneticin (Fujifilm Wako) or
589 hygromycin (Fujifilm Wako) and meropenem trihydrate (Fujifilm Wako) at the same
590 concentration as described above and incubated in the dark at 25 °C for a week. Gene
591 replacement was confirmed by PCR amplification using the primers listed in **Supplementary**
592 **table S3**.
593 Protoplasts of *A. oryzae* RIB40 and *A. flavus* NBRC114564 were transformed using
594 polyethylene glycol. First, 2.4×10^8 spores harvested from a 10-d-old culture on Czapek-Dox
595 agar were resuspended in 200 mL of Czapek-Dox broth and incubated at 30 °C at 160 rpm
596 for 18 h. The mycelium was then collected using miracloth (Merck, NJ, USA) and washed
597 once with distilled water, then incubated in 20 mL of a protoplast solution consisted of 10
598 mM phosphate buffer (pH6.0), 0.8 M NaCl, 10 mg/mL Lysing enzyme (Sigma-Aldrich, MO,
599 USA), 5 mg/mL Cellulase Onozuka R-10 (Yakult Pharmaceutical, Tokyo, Japan), and 2.5
600 mg/mL Yatalase (Takara, Shiga, Japan) at 30 °C with shaking at 83 rpm for 3 h. The
601 protoplasts were filtered through a mesh and centrifuged at 2000 × *g* and 4 °C for 5 min. The
602 pellet was washed once in 0.8 M NaCl and centrifuged at 2000 × *g* and 20 °C for 5 min, then
603 resuspended in 1.2 mL of solution 1 (Sol1: 9.35 g NaCl, 2 mL 1 M $CaCl_2$, 2 mL 1 M Tris-
604 HCl per 200 mL). Two handled fourty microliters of solution 2 (Sol2: 40% (w/v) PEG4000,
605 10 mL 1 M $CaCl_2$, 10 mL 1 M Tris-HCl per 200 mL) was added to the protoplast suspension
606 and the resulting mixture was dispensed 300 μL each into 14-mL round tubes A linearized
607 vector DNA was added to the mixture and held on ice for 30 min, then 1 mL of Sol2 was

608 added and held at 25 °C for 20 min. The protoplasts were washed once in 10 mL of Sol1, and
609 centrifuged at 2000 × *g* at 20 °C for 5 min. After the supernatant was discarded, the
610 protoplasts were resuspended in 300 μL of Sol1, then placed on a regeneration selection
611 medium consisted of Czapek-Dox agar supplemented with 5% (w/v) NaCl, 0.1 μg/mL
612 pyrithiamine hydrobromide (Sigma-Aldrich, MO, USA). The resulting plates were overlayed
613 with 5 mL of soft agar medium consisted of 5% (w/v) NaCl and 0.5% (w/v) agar, and
614 incubated at 30 °C for 4 d. The transformants grown on the regeneration selection medium
615 were isolated and transferred to Czapek-Dox agar containing 0.2 μg/mL pyrithiamine
616 hydrobromide and incubated at 30 °C for another selection. Gene replacement of the
617 transformants was confirmed by PCR amplification using the primers listed in
618 **Supplementary table S3**.
619
620 **Quantitative real-time PCR**
621 *C. fulvum* CF301 and *H. pulvinata* 414-3 were cultured on nylon membranes (GVS Japan) on
622 PDA at 25 °C in the dark for 1 and 2 weeks. The membranes were transferred to water agar
623 and then incubated in the same conditions for a week. *C. fulvum* colonies were sprayed with a
624 spore suspension of *H. pulvinata* (1×10^5 spores/mL) and checked for white colony growth
625 of *H. pulvinata* 1 week later. Total RNA was extracted from the mycelia using the RNeasy
626 Plant Mini Kit (Qiagen, Hilden, Germany). cDNA libraries were generated using SuperScript
627 IV VILO Master Mix with ezDNase Enzyme (ThermoFisher Scientific, Waltham, MA, USA)
628 according to the manufacturer's instructions. Primers for quantitative real-time PCR of 90–
629 150-bp fragments of cDNA were designed (**Supplementary table S3**), and the PCR was run
630 on the LightCycler 480 System (Roche, Basel, Switzerland) using the KAPA SYBR Fast
631 qPCR Kit (Nippon Genetics, Tokyo, Japan) according to the manufacturer's instructions.
632 Relative expression levels were calculated using the comparative CT ($2^{-\Delta\Delta CT}$) method (Livak

633 and Schmittgen, 2001). The data were normalized to the transcript level of the *H. pulvinata*
634 actin gene (**Supplementary table S3**). Transcript levels of target genes in each RNA sample
635 were measured for three independent experiments, each with two replicates.
636
637 **In-well assay and observation of mycoparasitism**
638 Green fluorescence protein (GFP)-expressing *C. fulvum* strain CF301gfp (Iida et al., 2018)
639 and *H. pulvinata* strains were cultured on PDA as mentioned above to prepare spore
640 suspensions of CF301gfp (5×10^4 spores/mL) and *H. pulvinata* (1×10^6 spores/mL). Then 50
641 µL of a spore suspension of CF301gfp and of each *H. pulvinata* strain were added to 100 µL
642 of MM broth without a carbon source (MM without sucrose) in each well of a 48-well plate.
643 At least 8 wells were used per treatment, and CF301gfp was used as the control. The plates
644 were incubated at 25 °C in the dark for 3 d. Hyphae were observed using a fluorescence
645 microscope BZ-X800 (KEYENCE, Osaka, Japan); lack of GFP fluorescence from *C. fulvum*
646 was correlated with the mycoparasitic activity of *H. pulvinata* using the hybrid cell count
647 system of the microscope. We named this simple method for detecting mycoparasitism the
648 IWAO method, derived from "in-well assay and observation" and in honor of the method's
649 developer, E. Iwao.
650
651 **Statistical analyses**
652 Means and standard deviations of number of spores, spore germination, and hyphal length
653 were calculated. Significant differences ($P < 0.05$) in the number of spores produced by the
654 different fungal strains, percentage spore germination and hyphal lengths of *C. fulvum*, and
655 colony diameter of *Aspergillus* strains were evaluated using either Tukey's test or Williams'
656 multiple comparison. Significant differences ($P < 0.05$) in gene expression levels were

657 determined using Welch's *t*-test followed by Bonferroni–Holm correction for multiple
658 testing. All data were analyzed in the program R version 4.0.3 (www.r-project.org).
659
660 **Data availability**
661 The genomic sequence of *H. pulvinata* 414-3 is available in the DDBJ repository under an
662 accession number BJKQ00000000 (BioProject: PRJDB8178) (Sushida et al., 2019). Other
663 fungal sequences shown in **Supplementary table S1** are available on the fungal genome
664 portal MycoCosm (mycocosm.jgi.doe.gov) of the Joint Genome Institute (Lawrence Berkeley
665 National Institute, Berkeley, CA, USA; Grigoriev, 2014) and in the DDBJ/EMBL/GenBank
666 repository (www.ncbi.nlm.nih.gov). Auxiliary data are available through Figshare
667 (doi.org/10.6084/m9.figshare.24559120.v1).
668
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676
677 **Conflict of interest**
678 The authors declare that they have no commercial or financial relationships that could be
679 construed as a potential conflict of interest.
680
681 **Author contributions**

682 YI designed the study and managed the research funds. KM, HS and YH performed qRT-
683 PCR and analyzed the data. KM, TaS and ON transformed fungal strains. ToS did the
684 electron microscopy and EI did the light microscopy. KM, TaS, YH and HN did the mass
685 spectrometry analyses. MZF and YN predicted the biosynthetic pathways and identified the
686 genes for enzymes. KM and HS did the phylogenetic analyses. KM and YI wrote the paper.
687 All authors contributed to the article and approved the submitted version.
688

689 **Supplementary figures**

690 Figure S1. Effect of deoxyphomenone on *Cladosporium fulvum* and tomato leaves.
691 Figure S2. Phylogenetic tree of fungal sesquiterpene cyclases.
692 Figure S3. Alignment and homology of the amino acid sequences of the predicted
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710 transformants.

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713 Figure S14. Reconciliation of gene trees with species tree based on *DPH* nucleotide and amino

714 acid sequences.

715 Figure S15. Hyphal contact between the mycoparasite *Hansfordia pulvinata* 414-3 and

716 *Aspergillus oryzae* RIB40.

717 Supplemental table S1. Fungal genome sequences used in this study.

718 Supplemental table S2. Amino acid sequences of *DPH* biosynthesis genes from *Hansfordia*

719 *pulvinata* and *Aspergillus* species.

720 Supplemental table S3. Primer sequences used in this study.

721

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923

924 **Figure legends**

925 **Figure 1. Deoxyphomenone produced by the mycoparasite *Hansfordia pulvinata* is an**
926 **antifungal sesquiterpene active against the tomato leaf mold pathogen *Cladosporium***
927 ***fulvum*.**

928 (**A**) Leaf mold symptoms (brown lesions) caused by *C. fulvum* and lesions covered by white
929 mycelia of *H. pulvinata* on a tomato leaf. (**B**) Scanning electron micrographs of hyphae of *H.*
930 *pulvinata* parasitizing hyphae of *C. fulvum.* Right: detail of area in dotted box in left image.
931 Arrow: bulge in hypha of *C. fulvum* caused by hyphae of *H. pulvinata*. (**C**) Germinated
932 spores of *C. fulvum* 24 h after treatment with 120 μM deoxyphomenone or with 1% methanol
933 (v/v) as a control (0 μM deoxyphomenone). Germination percentages are in parentheses.
934 Chemical structure of deoxyphomenone is also shown. (**D**) Antifungal activities of various
935 concentrations of deoxyphomenone in MM agar on mycelial growth of tomato pathogenic
936 fungi (*Cladosporium fulvum*, *Pseudocercospora fuligena* and *Fusarium oxysporum* f. sp.
937 *lycopersici*). Bars = 10 μm.

938

939 **Figure 2. Synteny of the deoxyphomenone biosynthetic (*DPH*) gene clusters conserved**
940 **in the mycoparasite *Hansfordia pulvinata* and *Aspergillus* section *Flavi*.**

941 (**A**) Gene clusters conserved in *H. pulvinata* and *Aspergillus*. The original gene IDs (DIP) and
942 names (*HpDPH1* to *HpDHP6*) of *H. pulvinata* are above the genes (detailed in
943 Supplementary Table S1). Protein annotations are indicated by color. The names of *HpDPH*
944 homologous genes in *Aspergillus* mentioned in the text are indicated in the arrows. Numbers
945 indicate the length of the genes and UTRs in base pairs. ORFs (black arrows) were detected
946 in the upstream region of *HpDPH6* in three *Aspergillus* species, and no homologous gene
947 sequence was found in the NCBI non-redundant database. (**B**) Predicted biosynthetic pathway
948 of deoxyphomenone. Intermediates were logically inferred. FPP, farnesyl pyrophosphate.

949

950 **Figure 3. Relative gene expression levels of the deoxyphomenone biosyntheticsis (*DPH*)**

951 **genes in the mycoparasite *Hansfordia pulvinata.***

952 A spore suspension of *H. pulvinata* 414-3 was sprayed on *Cladosporium fulvum* on water

953 agar (WA). *H. pulvinata* and *C. fulvum* were grown on PDA or WA as controls. Expression

954 was quantified by qPCR and normalized using those of *H. pulvinata* actin gene. Values are

955 the means of three biological replicates (± SD). Significant differences ($P < 0.05$) in

956 expression for a gene among the three treatments are indicated by different letters.

957

958 **Figure 4. Deoxyphomenone production by wild-type and knock-out mutants of**

959 ***Hansfordia pulvinata* and their antifungal activity.**

960 Wild-type 414-3 of *H. pulvinata*, *ΔHpDPH1* mutants (KO10, KO37) and *AoDHP1-*

961 complemented KO37 (CO10A) were cultured in MM broth for 2 weeks, and the filtered

962 culture filtrate collected to (**A**) quantify deoxyphomenone production in 414-3 and mutant

963 KO10 using LC-MS/MS. The retention time of the highest peak is given for each. (**B**) Time

964 course of deoxyphomenone production in 414-3 and mutant KO10. (**C**) Germination of

965 *Cladosporium fulvum* spores after 24 h of exposure to deoxyphomenone in culture filtrate

966 (CF) of 414-3 or KO10 (bars = 20 μm). Different letters in graph indicate significant

967 differences ($P < 0.05$) in germination among treatments. Values are means of three replicates

968 (± SD).

969

970 **Figure 5. Deoxyphomenone production and sporulation by *A. flavus* wild-type**

971 **NBRC114564 and Δ*AfDPH1* mutant strains and ectopic mutants.**

972 The wild-type, three Δ*AfDPH1* mutants, and two ectopic mutant strains were cultured in MM

973 broth or agar. Values are means of three replicates (± SD). (**A**) LC-MS/MS analysis of

974 deoxyphomenone in culture filtrates of the various strains. ND: not detected. (**B**) Number of
975 spores formed on agar. Asterisk indicates significant difference ($P < 0.05$) compared to the
976 wild-type in Williams' test. (**C**) Colony morphology of the wild-type, Δ*AfDPH1* mutant and
977 ectopic AfT32 mutant.
978
979 **Figure 6. Different effects of deoxyphomenone on spore germination of *Cladosporium***
980 ***fulvum* and spore production of two *Aspergillus* species.**
981 Wild-types *C. fulvum* CF301, *A. oryzae* RIB40 and *A. flavus* NBRC114564 were grown on
982 MM agar supplemented with deoxyphomenone. (**A**) Inhibition of spore germination of
983 *Cladosporium fulvum*. (**B**) Effects on sporulation of RIB40 and NBRC114564. Values are the
984 mean of three biological replicates. Error bars indicate standard deviations. Asterisks indicate
985 significant difference ($p < 0.025$) compared to control treatment (0 μM) by Williams' test.
986
987 **Figure 7. Phylogeny and sequence similarity of deoxyphomenone biosynthetic (*DPH*)**
988 **genes in *Hansfordia pulvinata* and *Aspergillus* species.**
989 The phylogenetic species tree based on the fungal genomic sequences (approximately 15 kb)
990 listed in Supplementary Table S1 was constructed using maximum likelihood. Asterisks
991 represent species that have been verified to produce deoxyphomenone. Sequence of
992 *Lyophyllum atratum* belonging to *Agaricomycetes* was used as an outgroup. *Aspergillus*
993 species were categorized into three groups based on the presence of *DPH* genes. The heatmap
994 represents the pairwise similarity (%) of the deduced amino acid sequences of the *DPH*
995 homologous genes.
996
997 **Figure 8. Proposed horizontal transfer of the deoxyphomenone biosynthetic (*DPH*) gene**
998 **cluster from ancestral *Aspergillus* species to the mycoparasite *Hansfordia pulvinata*.**

999 The phylogenetic species tree was based on 500 monocore genes (a single homolog in each
1000 of the species) from 60 fungal genomes and constructed using maximum likelihood, and
1001 horizontal gene transfer, duplication and loss events inferred in Supplementary figure S14
1002 were described. Horizontal gene transfer events for the *HpDPH1* (T1) and *DPH2/DPH4* (T2)
1003 genes are indicated by yellow stars. Proposed horizontal transfer of the *DPH* cluster from
1004 ancestral *Aspergillus* to *H. pulvinata* is highlighted by a yellow arrow. Gene loss is marked
1005 by an x on the branch. The three *Aspergillus* groups classified by the presence of *DPH* genes
1006 shown are the same as in Figure 7. Fungal species with either a complete or partial *DPH*
1007 cluster are in black, and those without the cluster are in grey. *Aspergillus* sections are given
1008 on the right. *Lyophyllum atratum* was used as an outgroup.

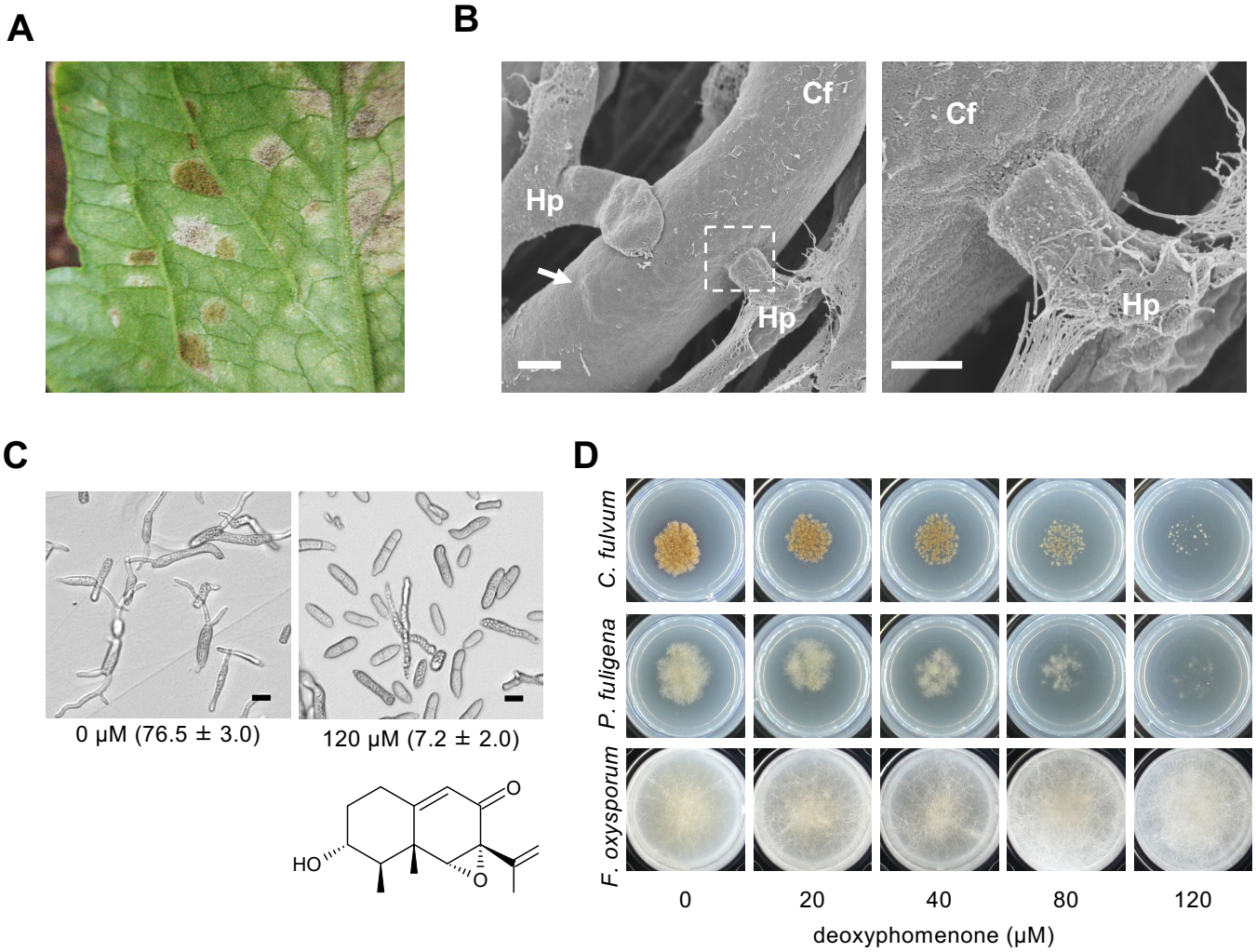
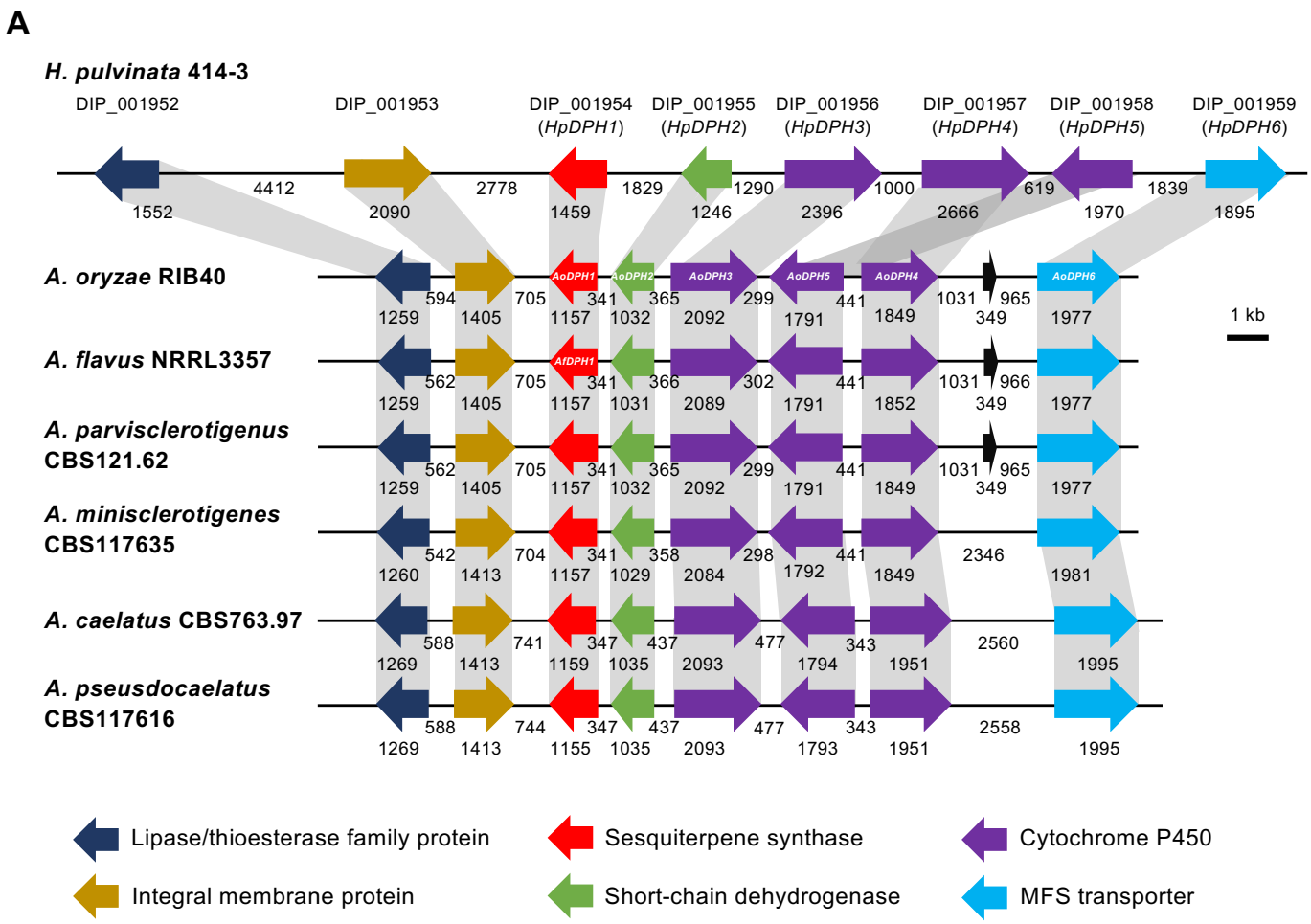

A
B
Cf
Hp
Hp
Cf
Hp
C
0 μM (76.5 ± 3.0)
120 μM (7.2 ± 2.0)
HO
O
O
D
C. fulvum
P. fuligena
F. oxysporum
0
20
40
80
120
deoxyphomenone (μM)

Figure 1


A
H. pulvinata 414-3
DIP_001952
DIP_001953
DIP_001954 (HpDPH1)
DIP_001955 (HpDPH2)
DIP_001956 (HpDPH3)
DIP_001957 (HpDPH4)
DIP_001958 (HpDPH5)
DIP_001959 (HpDPH6)
A. oryzae RIB40
A. flavus NRRL3357
A. parvisclerotigenus CBS121.62
A. minisclerotigenes CBS117635
A. caelatus CBS763.97
A. pseusdocaelatus CBS117616
AoDPH1
AoDPH2
AoDPH3
AoDPH5
AoDPH4
AoDPH6
AfDPH1
1 kb
Lipase/thioesterase family protein
Sesquiterpene synthase
Cytochrome P450
Integral membrane protein
Short-chain dehydrogenase
MFS transporter

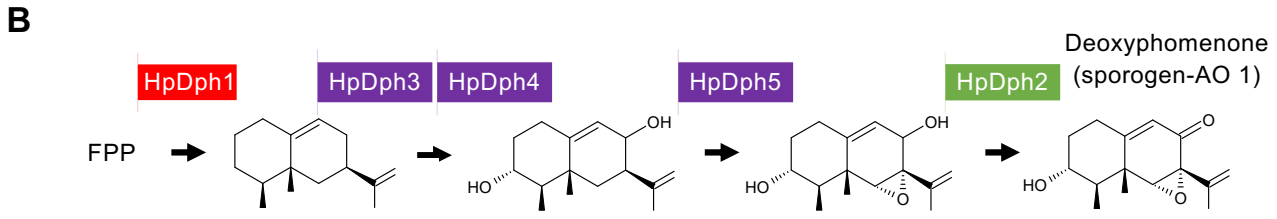

B
HpDph1
HpDph3
HpDph4
HpDph5
HpDph2
Deoxyphomenone (sporogen-AO 1)
FPP
OH
HO
O

Figure 2

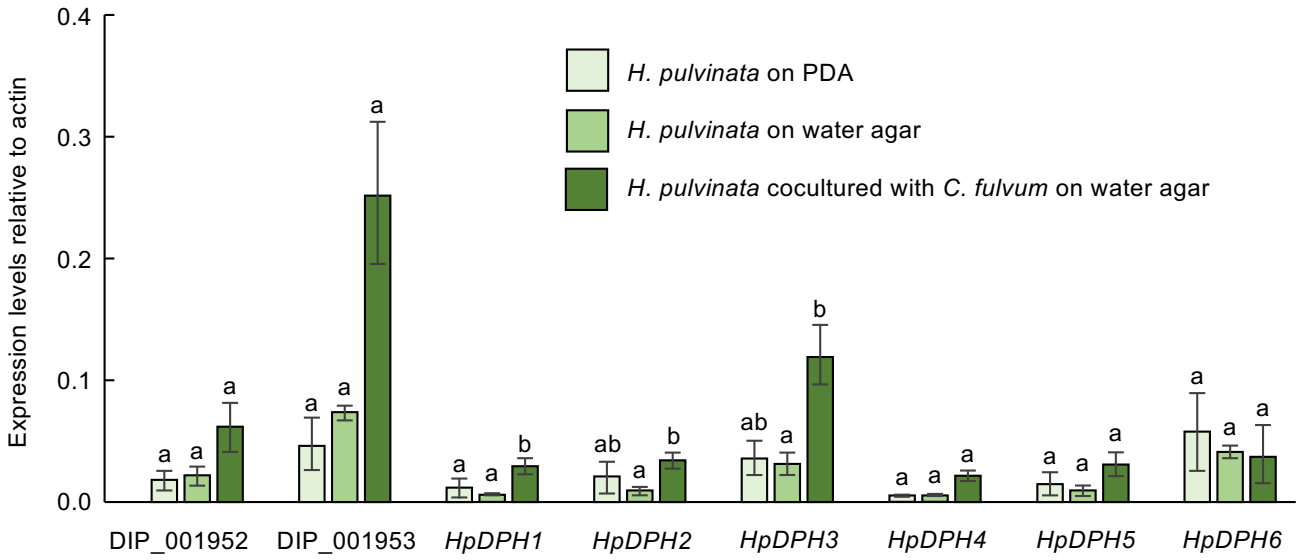

Expression levels relative to actin
0.4
0.3
0.2
0.1
0.0
H. pulvinata on PDA
H. pulvinata on water agar
H. pulvinata cocultured with C. fulvum on water agar
DIP_001952
DIP_001953
HpDPH1
HpDPH2
HpDPH3
HpDPH4
HpDPH5
HpDPH6

Figure 3

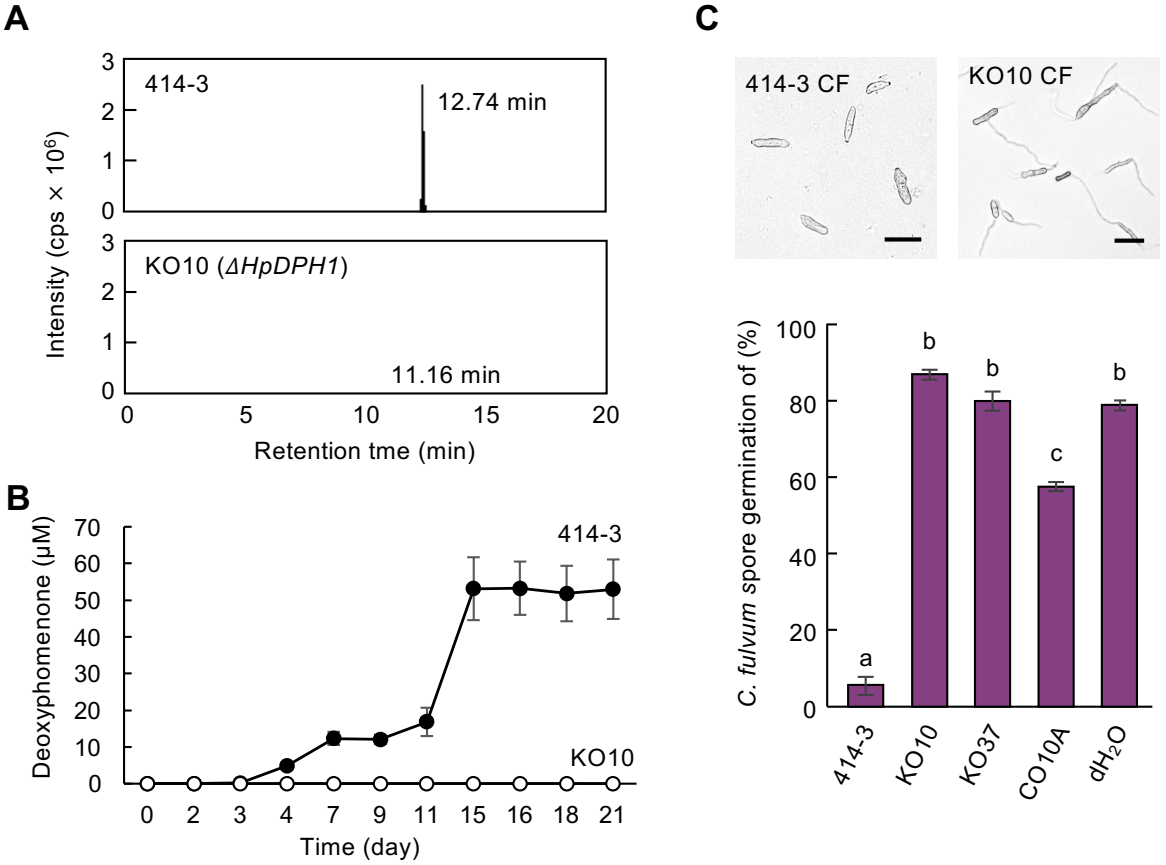

A
414-3
12.74 min
KO10 (ΔHpDPH1)
11.16 min
Intensity (cps × 10^6)
Retention tme (min)
B
414-3
KO10
Deoxyphomenone (μM)
Time (day)
C
414-3 CF
KO10 CF
C. fulvum spore germination of (%)
414-3
KO10
KO37
CO10A
dH2O

Figure 4

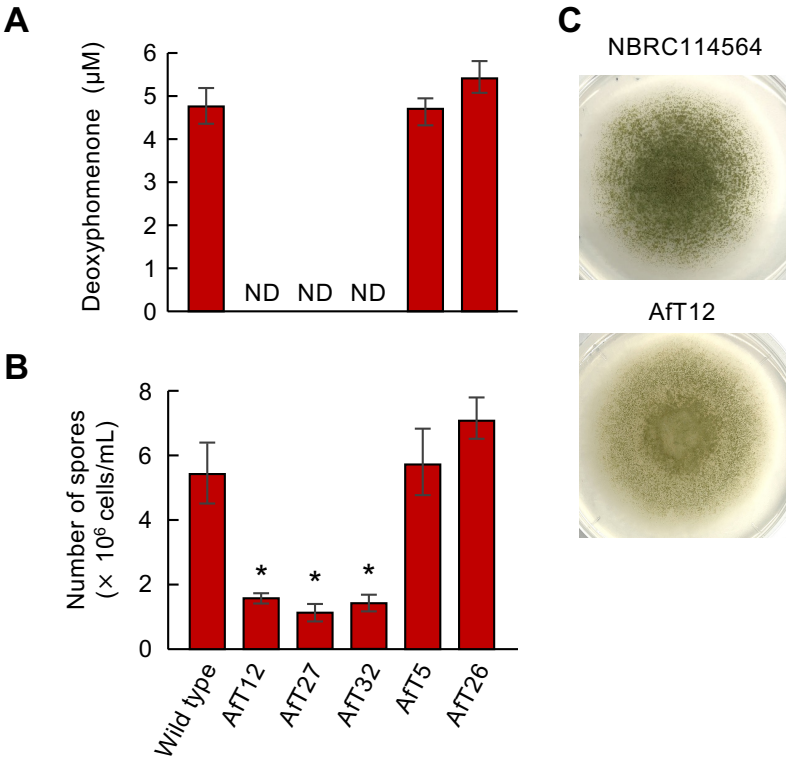

A
Deoxyphomenone (μM)
6
5
4
3
2
1
0
ND ND ND
B
Number of spores
(× 10^6 cells/mL)
8
6
4
2
0
*
*
*
Wild type
AfT12
AfT27
AfT32
AfT5
AfT26
C
NBRC114564
AfT12

Figure 5

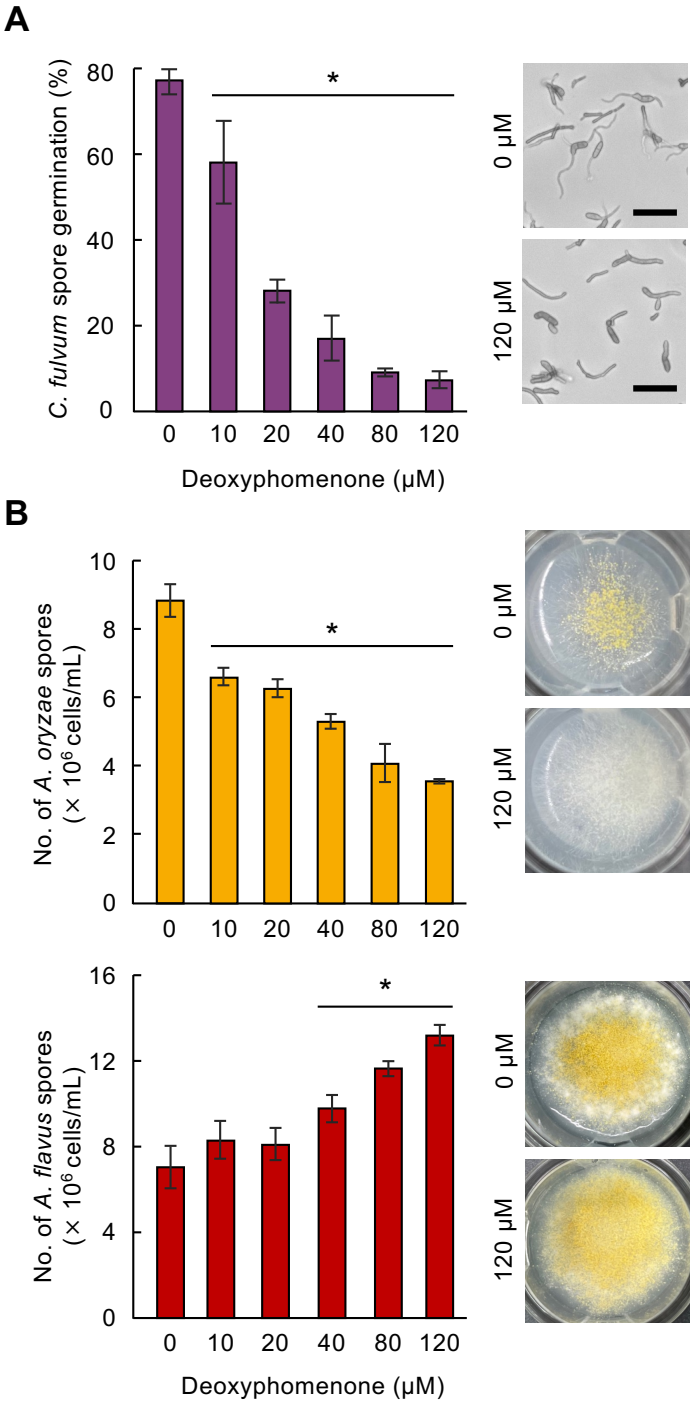

A
C. fulvum spore germination (%)
80
60
40
20
0
*
0 10 20 40 80 120
Deoxyphomenone (μM)
0 μM
120 μM
B
No. of A. oryzae spores (× 10^6 cells/mL)
10
8
6
4
2
0
*
0 10 20 40 80 120
0 μM
120 μM
No. of A. flavus spores (× 10^6 cells/mL)
16
12
8
4
0
*
0 10 20 40 80 120
Deoxyphomenone (μM)
0 μM
120 μM

Figure 6

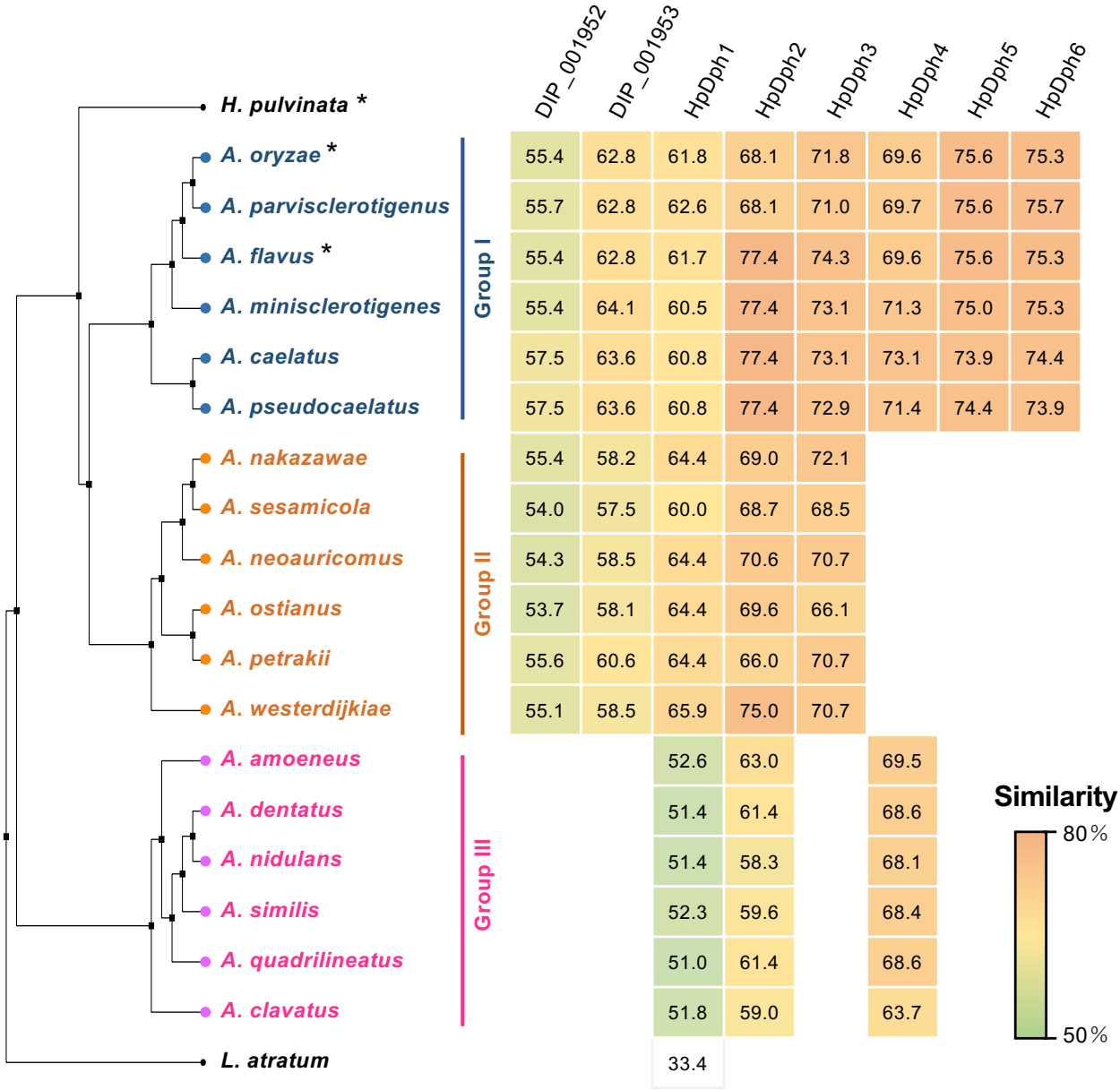

DIP_001952
DIP_001953
HpDph1
HpDph2
HpDph3
HpDph4
HpDph5
HpDph6
H. pulvinata *
A. oryzae *
A. parvisclerotigenus
A. flavus *
A. minisclerotigenes
A. caelatus
A. pseudocaelatus
A. nakazawae
A. sesamicola
A. neoauricomus
A. ostianus
A. petrakii
A. westerdijkiae
A. amoeneus
A. dentatus
A. nidulans
A. similis
A. quadrilineatus
A. clavatus
L. atratum
Group I
Group II
Group III
55.4 62.8 61.8 68.1 71.8 69.6 75.6 75.3
55.7 62.8 62.6 68.1 71.0 69.7 75.6 75.7
55.4 62.8 61.7 77.4 74.3 69.6 75.6 75.3
55.4 64.1 60.5 77.4 73.1 71.3 75.0 75.3
57.5 63.6 60.8 77.4 73.1 73.1 73.9 74.4
57.5 63.6 60.8 77.4 72.9 71.4 74.4 73.9
55.4 58.2 64.4 69.0 72.1
54.0 57.5 60.0 68.7 68.5
54.3 58.5 64.4 70.6 70.7
53.7 58.1 64.4 69.6 66.1
55.6 60.6 64.4 66.0 70.7
55.1 58.5 65.9 75.0 70.7
52.6 63.0 69.5
51.4 61.4 68.6
51.4 58.3 68.1
52.3 59.6 68.4
51.0 61.4 68.6
51.8 59.0 63.7
33.4
Similarity
80%
50%

Figure 7

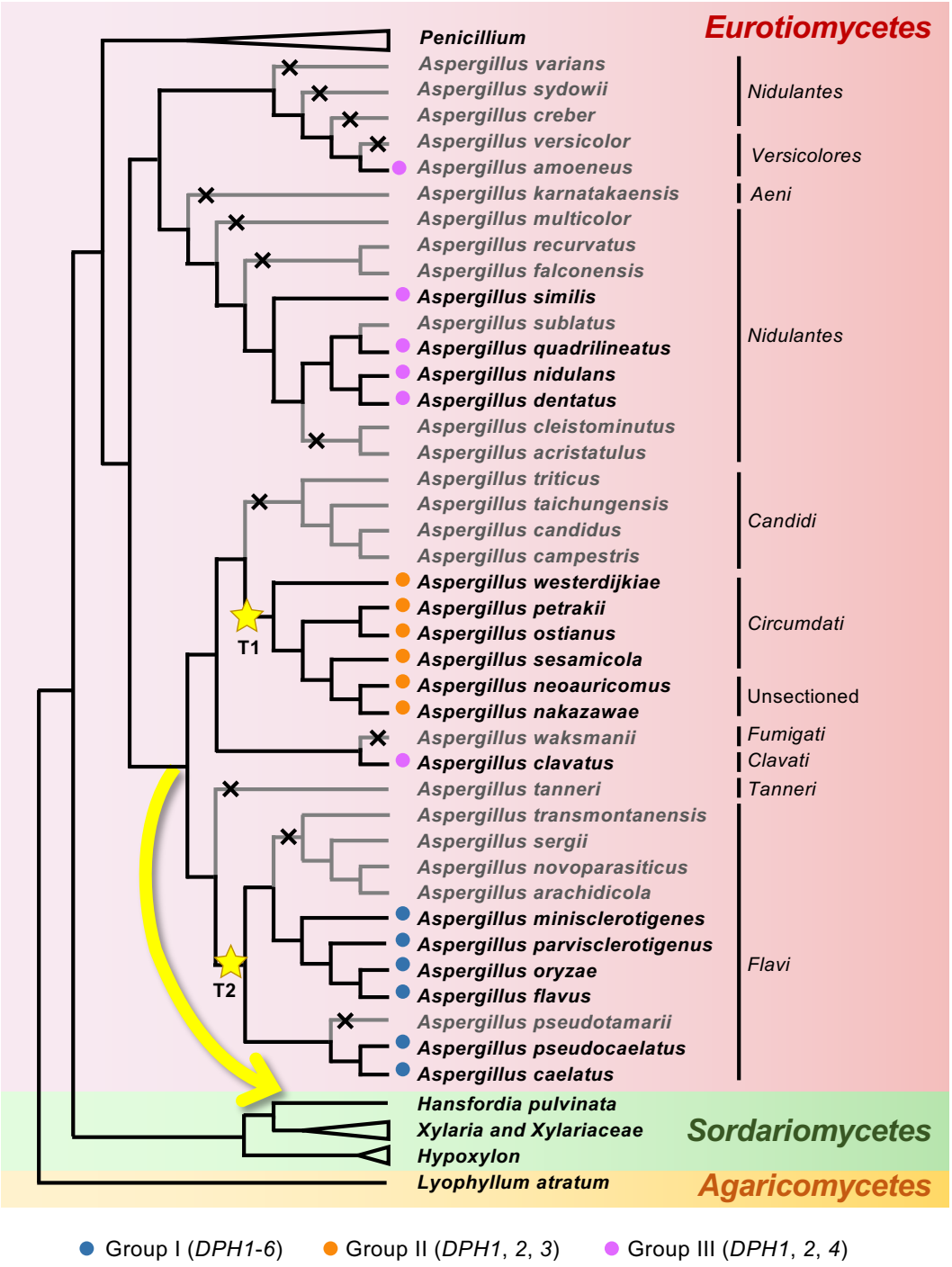

Eurotiomycetes
Penicillium
Aspergillus varians
Aspergillus sydowii
Aspergillus creber
Aspergillus versicolor
Aspergillus amoeneus
Aspergillus karnatakaensis
Aspergillus multicolor
Aspergillus recurvatus
Aspergillus falconensis
Aspergillus similis
Aspergillus sublatus
Aspergillus quadrilineatus
Aspergillus nidulans
Aspergillus dentatus
Aspergillus cleistominutus
Aspergillus acristatulus
Aspergillus triticus
Aspergillus taichungensis
Aspergillus candidus
Aspergillus campestris
Aspergillus westerdijkiae
Aspergillus petrakii
Aspergillus ostianus
Aspergillus sesamicola
Aspergillus neoauricomus
Aspergillus nakazawae
Aspergillus waksmanii
Aspergillus clavatus
Aspergillus tanneri
Aspergillus transmontanensis
Aspergillus sergii
Aspergillus novoparasiticus
Aspergillus arachidicola
Aspergillus minisclerotigenes
Aspergillus parvisclerotigenus
Aspergillus oryzae
Aspergillus flavus
Aspergillus pseudotamarii
Aspergillus pseudocaelatus
Aspergillus caelatus
Hansfordia pulvinata
Xylaria and Xylariaceae
Hypoxylon
Lyophyllum atratum
Nidulantes
Versicolores
Aeni
Nidulantes
Candidi
Circumdati
Unsectioned
Fumigati
Clavati
Tanneri
Flavi
T1
T2
Sordariomycetes
Agaricomycetes
Group I (DPH1-6)
Group II (DPH1, 2, 3)
Group III (DPH1, 2, 4)

Figure 8