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Depside and depsidone synthesis in lichenized fungi comes into focus through a genome-wide comparison of the olivetoric and physodic acid chemotype of *Pseudevernia furfuracea*

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19 **ABSTRACT**

20 Primary biosynthetic enzymes involved in the synthesis of lichen polyphenolic compounds
21 depsides and depsidones are Non-Reducing Polyketide Synthases (NR-PKSs), and
22 cytochrome P450s (CytP450). However, for most depsides and depsidones the corresponding
23 PKSs are unknown. Additionally, in non-lichenized fungi specific fatty acyl synthases (FASs)
24 provide starters to the PKSs. Yet, the presence of such FASs in lichenized fungi remains to be
25 investigated. Here we implement comparative genomics and metatranscriptomics to identify
26 the most likely PKS and FASs for the synthesis of olivetoric and physodic acid, the primary
27 depside and depsidone defining the two chemotypes of the lichen *Pseudevernia furfuracea*.
28 We propose that the gene cluster PF33-1_006185, found in both chemotypes, is the most
29 likely candidate for olivetoric and physodic acid biosynthesis. This is the first study to
30 identify the gene cluster and the FAS likely responsible for physodic and olivetoric acid
31 biosynthesis in a lichenized fungus. Our findings suggest that gene regulation and other
32 epigenetic factors determine whether the mycobiont produces the depside or the depsidone,
33 providing the first direct indication that chemotype diversity in lichens can arise through
34 regulatory and not only through genetic diversity. Combining these results and existing
35 literature, we propose a detailed scheme for depside/depsidone synthesis.

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37

38 **Key words**

39 Lichen-forming fungi, natural products, secondary metabolites, orsellinic acid derivatives,
40 chemosyndrome, biosynthetic gene clusters, fatty acyl synthases, cytochrome P450, PKSs

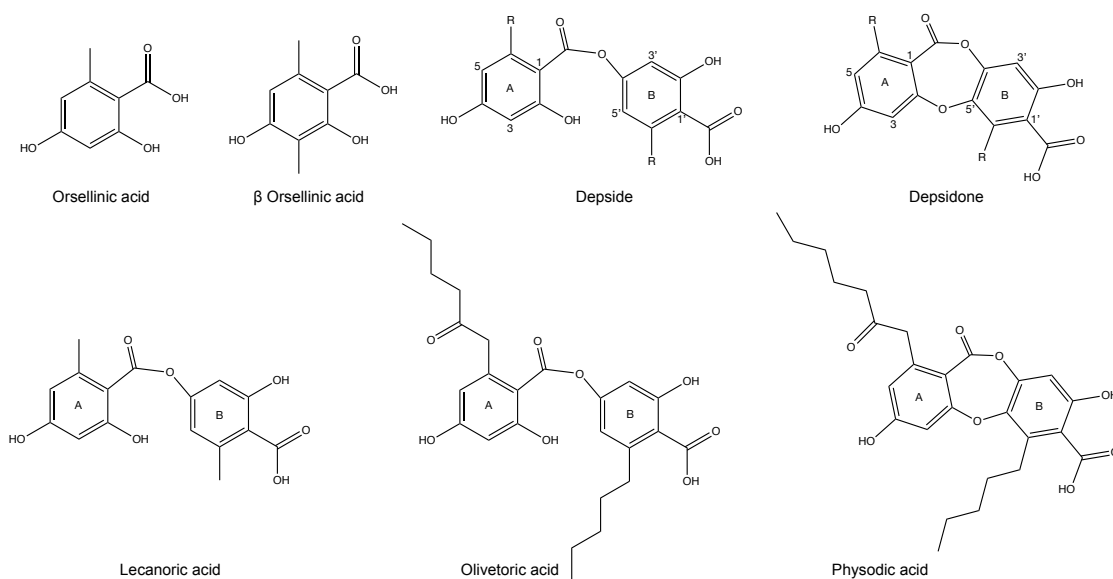
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42 **Introduction**

43

44 Depside and depsidones, the polyphenolic polyketides mostly synthesized by lichenized
45 fungi, are of significant pharmaceutical interest (Shukla *et al.*, 2010; Shrestha and St. Clair,
46 2013; Ingelfinger *et al.*, 2020). Depsides consist of two or sometimes three orcinol or β -
47 orcinol-derived aromatic rings joined by ester linkages; depsidones have an additional ether
48 linkage between the rings (Fig. 1). Additionally, depending on the starters used by the
49 polyketide synthases (PKSs) assembling their backbones, 3-7 carbon side chains may be
50 linked to the 6 and 6' carbons of the orcinol-derived rings. Together with other ring
51 modifications, side chains constitute the distinguishing features of different depsides and
52 depsidones. Although chemical proposals for deposite and depsidone biosynthesis go back
53 many decades (Seshadri, 1944; Elix *et al.*, 1987), the precise enzymatic steps of deposite and
54 depsidone synthesis still need to be elucidated. Furthermore, for most of the deposite and
55 depsidone metabolites of lichens the corresponding genes remain uncharacterized. This is
56 because fungi contain far more biosynthetic genes than known compounds (Meiser *et al.*,
57 2017; Calchera *et al.*, 2019). One way to connect metabolites to the associated genes is to
58 identify all the genomic regions with putative biosynthetic genes, and narrow down this
59 selection to the most likely gene cluster based on phylogenetic evidence, and other cluster
60 information, such as presence of particular genes. Long reads sequencing technologies
61 providing high quality contiguous genome assemblies have greatly facilitated this process.

62



63
64 **Figure 1.** Chemical structure of orsellinic acid and methyl-3-orsellinate (the monocyclic
65 precursors of depsides and depsidones), lecanoric acid, a depside, a depsidone, olivetoric acid,
66 and physodic acid. The letters and ring numberings used for the generic depside are the same
67 for all depsides and depsidones.

68
69 Fungal type I PKSs are iterative, and consist of several domains with defined
70 functions. Non-reducing, type I PKSs (NR-PKSs) contain KS (keto-synthase), AT
71 (acyltransferase), PT (product template), ACP (acyl carrier protein), and TE (thioesterase)
72 domains (Kroken *et al.*, 2003; Cox and Simpson, 2009). While NR-PKSs have long been
73 known to assemble and fold the carbon backbones of depsides and depsidones, their specific
74 roles in linking the rings have come to light only recently. A single PKS catalyzes the
75 formation and dimerization of phenolic rings to produce a depside (Armaleo *et al.*, 2011;
76 Kealey *et al.* 2021) while a cytochrome P450 is needed to catalyze the formation of an ether
77 bond between the depside rings to produce a depsidone (Armaleo *et al.*, 2011). The PKS
78 constructs and esterifies the two different rings using two ACP domains (Feng *et al.*, 2019).
79 The genes for PKS and cytochrome P450 (CytP450) are closely linked within the same
80 biosynthetic gene cluster (BGC). BCGs contain several genes involved in the synthesis of a
81 compound, e.g., the core biosynthetic PKS, redox enzymes, transporters, etc. A BGC is
82 named based on the type of backbone enzyme encoded by the core gene, e.g., a PKS cluster, a
83 terpene cluster, etc.

84 Apart from the above-stated genes, studies with non-lichen-forming fungi indicate
85 that specific fatty acyl synthases (metabolite FASs) play significant roles in metabolite
86 synthesis by providing the appropriate acyl chain starters to some PKSs (Hitchman *et al.*,
87 2001; Watanabe and Townsend, 2002; Smith and Tsai, 2007). Inactivation of metabolite
88 FASs may inhibit secondary metabolite synthesis even when the corresponding PKS and
89 BGC remain functional (Brown *et al.*, 1996). However, the role of metabolite FAS in
90 providing the starters for depsides and depsidones in lichen-forming fungi has not been
91 investigated, despite the fact that the primary starters of orcinol depsides and depsidones are,
92 besides the C2 doublet from AcetylCoA, C4, C6, and C8 acyl chains (Culberson and
93 Culberson, 1976). After polyketide assembly and cyclization, the resulting ring sidechains
94 will respectively be 1, 3, 5, 7 carbons long. Recently and coincidentally, the NR-PKS from
95 the lichen *Pseudevernia furfuracea* that we identify in this work as the likely producer of the
96 depside olivetoric acid was reported to produce the depside lecanoric acid when
97 heterologously expressed in yeast (Kealey *et al.*, 2021). Yet in nature lecanoric acid has never
98 been reported from *P. furfuracea*. The difference between lecanoric and olivetoric acid is that
99 the former has a methyl group on each ring whereas the latter has as a C5 side chain on one
100 ring and a C7 side chain on the other (Fig. 1). We integrate this apparent discrepancy with
101 other data to highlight the central role that lichen short chain FASs are likely to play in
102 providing the side chains common in orcinol depsides and depsidones.

103 In this study we implemented a long-read-based genomic approach to better
104 understand the mechanism of depside/depsidone synthesis in lichen-forming fungi. We chose
105 *Pseudevernia furfuracea* as our study system, because it is a textbook example of
106 chemosyndrome variation in a lichen-forming fungus (Culberson *et al.*, 1977; Halvorsen and
107 Bendiksen, 1982; Kosanić *et al.*, 2013). This lichen consists of two naturally occurring
108 chemotypes, one synthesizing an orcinol depside (olivetoric acid), and the other the
109 corresponding depsidone (physodic acid), and thus constitutes an ideal model to study
110 depside/depsidone synthesis, and the causes of chemotype diversity. Both chemotypes also
111 synthesize the β -orcinol depside atranorin, common in many lichens. Specifically, we aim to
112 answer the following questions: 1) Do the depside and the depsidone producer contain the
113 same number of BGCs? 2) Which BGC/s are likely responsible for the production of depside
114 olivetoric acid and the depsidone physodic acid in *P. furfuracea* chemotypes? 3) Are there
115 homologs of metabolite FASs in the lichen-forming fungal genome? 4) Can we integrate the
116 available data to provide a detailed scheme of orcinol depside/depsidone biosynthesis in
117 lichens?

118 **Materials and methods**

119 **Identification of chemotypes**

120 We used high performance liquid chromatography (HPLC) to investigate the chemotype of *P.*
121 *furfuracea*. For this, we collected several samples of *P. furfuracea* and performed HPLC
122 analysis using the protocol from Feige et al. and Benatti et al. (2013). Firstly, small thallus
123 pieces were extracted for 1 hour at room temperature in 200 μ l of methanol. From this, 150 μ l
124 of the extract of each sample was centrifuged 1 min at 800 rpm through a Pall Acroprep
125 Advance 0.2 μ m polytetrafluoroethylene filter plate and then diluted 10-fold with methanol.
126 The samples were analyzed on an Agilent 1260 quaternary system with a quaternary pump, an
127 incorporated degasser and using an Agilent Poroshell 120 EC-C18 column (2.7 μ m, 3.0 x 50
128 mm). Substances were separated at 30°C using two solvent systems and a flow rate of 1.4
129 ml/min. Solvent A is Aqua Bidest, 30% methanol and 0.0658% trifluoroacetic acid, and
130 solvent B is 100% methanol. The HPLC system was equilibrated to solvent A for 2 min and
131 2 μ l of extract was injected automatically after a needle wash. The runs continued isocratically
132 for 0.18 min, solvent B was increased to 58% within 5 min, then increased to 100% within the
133 next 5 min and isocratically maintained for 0.82 min. The runs ended with solvent A being
134 increased back to 100% within 0.5 min. After the run the column was flushed for two minutes
135 before the next run. Compounds were detected with a diode array detector (DAD) at 210, 254,
136 280 and 310 nm. The retention times and spectra (λ = 190-650 nm with 2 nm steps) were
137 compared against a library of authentic products derived under identical conditions using the
138 Agilent OpenLAB CDS ChemStation software. We then selected one sample of each
139 chemotype for genome sequencing (Supplementary Table S1).

140

141 **DNA extraction and genome sequencing**

142 Lichen thalli were thoroughly washed with sterile water, and checked under the
143 stereomicroscope for the presence of possible contamination. DNA was extracted from both
144 samples using a CTAB-based method (Cubero and Crespo, 2002). DNA concentration was
145 measured with a Qubit fluorometer (dsDNA BR, Invitrogen). 4.1 μ g and 7.4 μ g DNA for the
146 physodic- and olivetoric acid chemotype, respectively, were sent to Novogene Hong Kong for
147 PacBio library preparation and sequencing on two separate SMRT cells, one for each
148 chemotype.

149

150 **Genome assembly and annotation**

151 PacBio metagenomes were assembled using the long-read based assembler metaFlye v2.3.1
152 (Kolmogorov *et al.*, 2019). Reads were filtered for length (>2000 kb fragments only) and
153 assembly was optimized for minimal read overlap of 3 kb, and an estimated combined
154 metagenome size of 120 Mb. The assembled genome was polished twice using the software
155 Arrow from the SMRTlink suite v. 5.0.1.9585 (Walker *et al.*, 2014). The resulting contigs
156 were then scaffolded with SSPACE-LongRead v1.1 (Boetzer and Pirovano, 2014).
157 Ascomycota contigs were then identified in the metagenomic assembly using Diamond
158 v0.8.34.96 BLASTx using the more-sensitive mode for longer sequences and a default e-value
159 cut-off of 0.001 against the custom database. The Diamond results were then parsed in
160 MEGAN68 v.6.7.7 using max expected set to 1E-10 and the weighted lowest common
161 ancestor (LCA) algorithm. All contigs assigned to Ascomycota were exported to represent the
162 *P. furfuracea* mycobiont. Assembly indicators such as number of contigs, total length and
163 N50 were accessed with Assemblathon v2 (Table 1). Genome completeness was estimated
164 based on evolutionarily-informed expectations of gene content with BUSCO v.4.0
165 (Benchmarking Universal Single-Copy Orthologs) (Simão *et al.*, 2015). The genomes are
166 deposited in GenBank under accessions xx and xx.

167

168 **Identification and Annotations of Biosynthetic Gene Clusters**

169 Gene prediction, functional annotation and prediction of BGCs in both *P. furfuracea*
170 chemotype assemblies were performed with scripts based on the funannotate pipeline
171 (Palmer and Stajich, 2019) and antiSMASH (antibiotics & SM Analysis Shell, v5.0)
172 (Medema *et al.*, 2011; Blin *et al.*, 2019). First, the repetitive elements were masked in the
173 assembled genomes (using funannotate), followed by gene prediction using BUSCO2 to train
174 Augustus and self-training GeneMark-ES. Functional annotation was then automatically
175 carried out with InterProScan, EggNOG-mapper and BUSCO ascomycota_odb10 models.
176 Secreted proteins were predicted using SignalP as implemented in funannotate 'annotate'
177 command. The interproscan, antismash and phobius results were automatically generated.

178

179 **Identification of homologous BGCs**

180 Homologous clusters between the two *P. furfuracea* chemotypes were identified by
181 performing reciprocal blast between the core genes of the BGCs of both genomes. For this,
182 first the core genes from the predicted BGCs of one chemotype were used as database and the
183 core genes of the BGCs from the other chemotype as query. The process was then repeated

184 using the other chemotype as database. The homology between the clusters was then
185 confirmed based on sequence similarity and the most similar hit of the core gene in the
186 MIBiG v2 (Minimum Information about a Biosynthetic Gene cluster; (Kautsar *et al.*, 2020))
187 database (Supplementary table S2).

188 Homologous clusters were visualized using synteny plots as implemented in
189 Easyfig v2.2.3 (Sullivan *et al.*, 2011). The GBK input files for Easyfig were generated with
190 seqkit v0.10.1 (Shen *et al.*, 2016) and the seqret tool from EMBOSS v6.6.0.0 (Rice *et al.*,
191 2000). Easyfig was run with tblastx v2.6.0+, a minimum identity value of 90 and a minimum
192 length of 50 to draw the blast hits (Kjærboelling *et al.*, 2018). Clusters were manually matched
193 for orientation so that the core gene were oriented in the same direction. For six BGCs, no
194 corresponding cluster was detected in the other chemotype (Table 2, Supplementary Table
195 S2).

196

197 **Phylogenetic analyses**

198 NR-PKSs have been divided into nine groups based on protein sequence similarity and PKS
199 domain architecture (Ahuja *et al.*, 2012; Liu *et al.*, 2015; Kim *et al.*, 2021). We took
200 representative PKSs from each group (amino acid sequences) and added the amino acid
201 sequences of the eight *NR-PKSs* from *P. furfuracea*. The dataset includes 107 PKS sequences
202 from *Cladonia borealis*, *C. grayi*, *C. macilenta*, *C. metacorallifera*, *C. rangiferina*, *C.*
203 *uncialis*, *Pseudevernia furfuracea* and *Stereocaulon alpinum*. Sequences were aligned using
204 MAFFT as implemented in Geneious v5.4. Gaps were treated as missing data. The maximum
205 likelihood search was performed on the aligned amino acid sequences with RAxML-HPC
206 BlackBox v8.1.11 (Stamatakis, 2006, 2014) on the Cipres Scientific gateway (Miller *et al.*,
207 2010).

208

209 **Candidate cluster for physodic- and olivetoric acid synthesis**

210 In addition to the phylogenetic evidence, we implemented several criteria to select the
211 candidate cluster for depside/depsidone synthesis in *P. furfuracea*: 1) it must be present in
212 both chemotypes, (presence in both chemotypes is expected as the basic structure of physodic
213 and olivetoric acid is same except that physodic acid contains an additional ether bond (Fig.
214 1)) 2) it must contain a *NR-PKS* (the non-reduced backbone of lichen depsides/depsidones
215 suggests that the *PKSs* involved in their synthesis are *NR-PKSs*), and 3) the *NR-PKS* must
216 contain two ACPs (the presence of two ACPs has been associated with depside production in
217 fungi (Feng *et al.*, 2019; Lünne *et al.*, 2020), and is a typical feature of lichen-forming fungal

218 *NR-PKSs* involved in depside/depsidone synthesis (Armaleo *et al.*, 2011; Pizarro *et al.*,
219 2020)). Additionally, in the physodic acid producer the candidate BGC must contain a
220 *CytP450* which produces depsidones by forming the ether bond between the two orsellinic
221 rings of the depside (Armaleo *et al.*, 2011).

222 Summarizing, the following criteria were used for the identification of olivetoric-
223 /physodic acid BGC: 1) the candidate BGC should be homologous and present in both
224 chemotypes, 2) presence of a *CytP450*, and 3) presence of two ACP domains in the *PKS* as
225 the ring dimerization of orsellinic acid precursors into a depside involves two ACP domains
226 (Feng *et al.*, 2019; Lünne *et al.*, 2020).

227

228 **Identification of HexA and HexB**

229 Metabolite FASs consist of a HexA/HexB multienzyme complex (Brown *et al.*, 1996;
230 Hitchman *et al.*, 2001). Homologous of *HexA* and *HexB* were identified by blasting (blastN)
231 the *HexA* and *HexB* homologs of *Cladonia grayi* (CLAGR_008938-RA and
232 CLAGR_008939-RA, available at [https://mycocosm.jgi.doe.gov/cgi-](https://mycocosm.jgi.doe.gov/cgi-bin/browserLoad/?db=Clagr3&position=scaffold_00085:34887-93856)
233 [bin/browserLoad/?db=Clagr3&position=scaffold_00085:34887-93856](https://mycocosm.jgi.doe.gov/cgi-bin/browserLoad/?db=Clagr3&position=scaffold_00085:34887-93856)) against the genomes
234 of both chemotypes.

235

236 **Metatranscriptome analyses and quantification of *PKS*, *CytP450* and *HexA* and *HexB*** 237 **transcripts**

238 The details of RNA isolation and transcriptome extraction are given in Meiser *et al.* (2017).
239 Briefly, for RNA isolation, whole lichen thalli were collected and stored directly in RNAlater
240 (Sigma-Aldrich Chemie GmbH, Munich, Germany). RNA was isolated from both chemotypes
241 of *P. furfuracea* by using the method described by Rubio-Piña & Zapata-Pérez (2011) after
242 blotting the thalli dry and grinding them in liquid nitrogen with a mortar and pestle. The
243 isolated poly-A⁺ RNA was further purified with the RNeasy MinElute Clean-up Kit (Qiagen,
244 Hilden, Germany), and sequenced (250 bp paired-end reads) on Illumina MiSeq at StarSeq
245 (Mainz, Germany).

246 The BGC for depside/depsidone biosynthesis in each chemotype contains 10
247 genes including one *Pfur33-1_006185*, one *CytP450*, and a monooxygenase (see below). The
248 other seven code for unidentified proteins. We used transcriptome data to check which genes
249 in this cluster are transcriptionally active. For this, we first indexed the sequence of interest
250 using bowtie and then aligned it to the transcripts (both paired and unpaired reads) using

251 tophat v2 (Kim *et al.*, 2013). To make the counts comparable between chemotypes we used
 252 RPKM normalization of the read counts (Mortazavi *et al.*, 2008), accounting for sequencing
 253 depth and gene length (Oshlack and Wakefield, 2009; Robinson and Oshlack, 2010; Dillies *et*
 254 *al.*, 2013). The normalization for sequencing depth was performed by dividing the counts by
 255 the raw read count of the given gene with the total number of reads in each sample. The
 256 resulting number was then divided by gene length in kilobases to obtain RPKM normalized
 257 counts.

258

259 Results

260

261 Genomes of the *P. furfuracea* chemotypes

262 The number of reads for each sample retained after quality and length filtering is given in
 263 Table 1. The reference genome of the PacBio-based *P. furfuracea* physodic acid chemotype
 264 (NCBI acc. no. XXX) is ~34 Mb in length and has a completeness of 96% according to
 265 BUSCO (details in Table 1). The genome of the olivetoric acid chemotype (NCBI acc. no.
 266 XXX) is ~37 Mb in length and has a completeness of 92% according to BUSCO (details in
 267 Table 1).

268

Table 1. Genome statistics and GB accession numbers of the two chemotypes of *P. furfuracea*

		Physodic acid chemotype	Olivetoric acid chemotype
Sequencing report	data repository	XXX	XXX
	Subreads bases(G)	8.182	8.122
	Average subreads length	9563	9325
	N50 raw reads	12855	12479
Assembly stats	# scaffolds	104	53
	cds	11199	10480
	size (Mb)	34.2	37.3
	N50	632kb	1.6 Mb
	% completeness	96	92
	homologous clusters		51
	chemotype specific clusters	5	1
BGCs summary	R-PKSs	19	14
	NR-PKSs	4	4
	hybrid	10	8
	T3-PKS	1	1
	NRPS	4	4
	NRPS-like	10	<u>12</u>

Terpenes	6	<u>6</u>
indole	2	<u>2</u>

269

270 **Table 1.** Genome statistics and GB accession numbers of the two chemotypes of *P.*

271 *furfuracea*. Biosynthetic gene clusters (BGCs) predicted by antiSMASH are also given.

272 PKS=polyketide synthase, R-PKS=reducing PKS, NR-PKS=non-reducing PKS T3 PKS=xx,

273 hybrid=xx, NRPS=non-ribosomal peptide synthetase.

274

275 **Predicted BGCs**

276 A total of 51 homologous BGCs were present in both chemotypes: 14 clusters with reducing

277 *PKSs* (*R-PKS*), eight clusters with *NR-PKSs*, one cluster with type III *PKS*, seven hybrid

278 clusters, 14 clusters with *NRPS* or *NRPS*-like genes, five clusters with terpene synthase, and

279 two clusters with indole synthase as a core gene (Supplementary Table S2). Six BGCs, were

280 found only in one of the two chemotypes (Table 2). Five BGCs were only present in the

281 physodic acid chemotype (four BGCs with a *R-PKS*, a hybrid cluster with a *R-PKS* and a

282 *NRPS*, and a cluster with terpene synthase as the core gene), and one BGC with terpene

283 synthase as the core gene was present only in the olivetoric acid chemotype (Table 2).

284

Table 2. Number of raw DNA reads (nucleotides) normalized by number of reads and gene length aligned to the core genes of the clusters detected in only one chemotype

Clusters detected in only one chemotype					
cluster number	name of the cluster	detected in	core gene	normalized read count in physodic acid chemotype	normalized read count in olivetoric acid chemotype
52	Region 7.2	Physodic acid chemotype	R-PKS	24.66	0
53	Region 25.1	Physodic acid chemotype	R-PKS	11.50	0
54	Region 60.2	Physodic acid chemotype	hybrid	24.44	0
55	Region 65.1	Physodic acid chemotype	R-PKS	18.30	0
56	Region 27.1	Physodic acid chemotype	terpene	59.99	0
57	Region 10.1	olivetoric acid chemotype	terpene	0	133.94
Clusters present in both chemotypes					
1	Region 12.3	both chemotypes	NR-PKS	18.19	47.66
2	Region 18.1	both chemotypes	NR-PKS	16.43	56.19
3	Region 44.1	both chemotypes	NR-PKS	29.05	43.41
4	Region 33.1	both chemotypes	NR-PKS	37.08	42.14
9	Region 10.1	both chemotypes	R-PKS	20.14	45.88
11	Region 16-1	both chemotypes	R-PKS	25.53	48.84
12	Region 2.4	both chemotypes	R-PKS	33.33	42.85
15	Region 9.2	both chemotypes	R-PKS	35.00	39.86
6	Region 9.3	both chemotypes	R-PKS	23.87	41.47
5	Region 2.2	both chemotypes	R-PKS	26.24	53.15

44	Region 38.1	both chemotypes	terpene	40.34	107.15
45	Region 33.2	both chemotypes	terpene	83.56	119.52
46	Region 2.3	both chemotypes	terpene	37.49	114.33
47	Region 12.2	both chemotypes	terpene	50.63	149.93
48	Region 60.1	both chemotypes	terpene	51.24	85.36

285

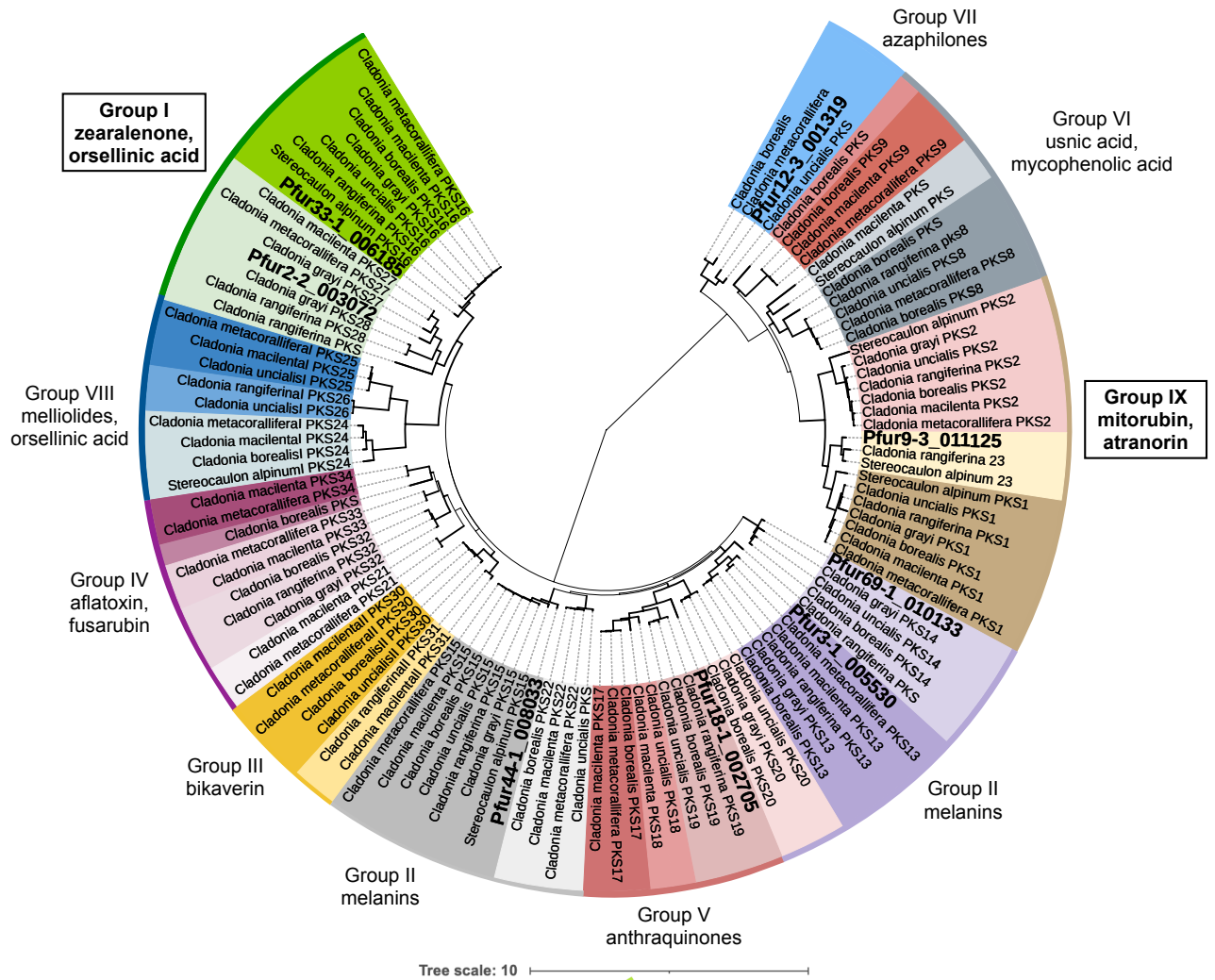
286 **Table 2.** Properties of the clusters detected in only one chemotype including the core gene
287 and its length, number of raw reads (nucleotide) normalized read counts (by number of reads
288 and gene length (RPKM approach)) aligned to the core genes of the clusters detected in only
289 one chemotype.

290

291 **Phylogenetic analyses**

292 Out of eight *NR-PKSs*, only two, *Pfur33-1_006185* and *Pfur2-2_003072*, grouped into
293 phylogenetic group I, whose *PKSs* are involved in the synthesis of orcinol derivatives (Fig. 2),
294 like grayanic, olivetoric and physodic acid. Of these *PKSs*, *Pfur33-1_006185* was closely
295 related to *PKS16*, the *PKS* associated with grayanic acid biosynthesis from *Cladonia grayi*,
296 whereas *Pfur2-2_003072* was closely related to *PKS27*. The *Pfur33-1_006185* cluster also
297 contains a *CytP450* next to the *NR-PKS* in an arrangement analogous to that in the *PKS16*
298 cluster in *C. grayi*.

299



300

301 **Figure 2.** A maximum likelihood tree of 107 NR-PKSs amino acid sequences from six
 302 *Cladonia* spp., *Stereocaulon alpinum* and eight *P. furfuracea*. Branches is bold indicate the
 303 bootstrap support of >70%. Different colors indicate different PKSs. PKS families are based
 304 on Kim et al. (2021). PKSs of *P. furfuracea* are indicated in bold and with larger fonts.

305

306 Selection of the candidate cluster for physodic acid and olivetoric acid

307 We complemented the phylogenetic evidence with the other characteristics of the cluster to
 308 select the possible olivetoric/physodic acid cluster. We found eight clusters containing a *NR-*
 309 *PKS* that were present in both chemotypes (Supplementary Table 2, Table 3). Of these, only
 310 one cluster, cluster 4 (Table 3), contained a *NR-PKS* with two ACP domains and a *CytP450* in
 311 the cluster. The domains of this PKS are – SAT-KS-AT-ACP-ACP-TE (Fig. 3). The most
 312 similar *PKS* to this is the *PKS* linked to grayanic acid biosynthesis, PKS16. We therefore
 313 propose cluster 4 to be the most likely candidate for olivetoric-/physodic acid biosynthesis in
 314 *P. furfuracea*. The cluster has an almost identical structure in both chemotypes, with 10 genes

315 including a *NR-PKS*, a *CytP450* and a monooxygenase (Fig. 3). The protein products of the
 316 remaining seven genes are unidentified.

317

Table 3. NR-PKS clusters detected in both chemotypes of *P. furfuracea*, the PKS domains and other genes present in the cluster

cluster number	PKS	compound defining the group (Kim et al 2021)	PKS category (Kim et al 2021)	PKS domains	total genes in the cluster	identified genes in cluster	Most similar known PKS (MIBiG)	coverage & similarity to MIBiG cluster
1	Pfur12-3_001319	VII (azaphilones, monascorubrin)	not known	SAT-KS-AT-ACP cMT-TD	12	Regulatory gene, NRPKS	monascorubrin	95%, 58%
2	Pfur18-1_002705	V (anthraquinones)	PKS20	SAT-KS-AT-PT-ACP	16	metallo-beta-lactamase family protein, NRPKS, halogenase	RES-1214-2	61%, 100%
3	Pfur44-1_008033	II (melanins)	PKS15	SAT-KS-AT-PT-ACP ACP-TE	11	PKS, dehydrogenase/reductase (KR)	naphthalene	99.5%, 53%
4	Pfur33-1_006185	I (zearalenone, orsellinic acid)	PKS16	SAT-KS-AT-ACP ACP-TE	10	cyt P450, PKS, monooxygenase	grayanic acid	99%, 73%
5	Pfur2-2_003072	I (zearalenone, orsellinic acid)	PKS27	SAT-KS-AT-PT-ACP-TE	13	Omethyltransferase, cyt P450, crotonyl-CoA reductase / alcohol dehydrogenase, red-PKS, NRPKS, GATase 7	grayanic acid	100%, 38%
6	Pfur9-3_011125	IX (mitorubin, atranorin)	PKS23	SAT-KS-AT-PT-ACP cMT	11	alkyl hydroperoxide reductase/ Thiol specific, PKS, cyt P450, drug resistance transporter	ascochlorin cluster (<i>Acremonium egyptiacum</i>)	100%, 38%
7	Pfur3-1_005530	II (melanins)	PKS13	SAT-KS-AT-PT-ACP-ACP-TE	13	serine/threonine protein kinase, Drug resistance transporter, monooxygenase FAD-binding, NRPKS, short-chain dehydrogenase/reductase SDR, O-methyltransferase, transcription regulator		100%, 48%
8	Pfur69-1_010133	II (melanins)	PKS14	SAT-KS-AT-PT-ACP-ACP-TE	9	Drug resistance transporter, O-methyltransferase, halogenase, monooxygenase FAD-binding, adh_short (DH-KR), NRPKS, monooxygenase FAD-binding	6-hydroxymellein (<i>Cladonia uncialis</i>)	99%, 78%

318

319 **Table 3.** Properties of the *NR-PKS* clusters detected in both the chemotypes of *P. furfuracea*.

320 NR-PKS= non-reducing PKS. The cluster in bold, in the box (cluster 4 containing *Pfur33-*

321 *I_006185*), is the likely cluster for depside/depsidone synthesis. The domain acronyms stand

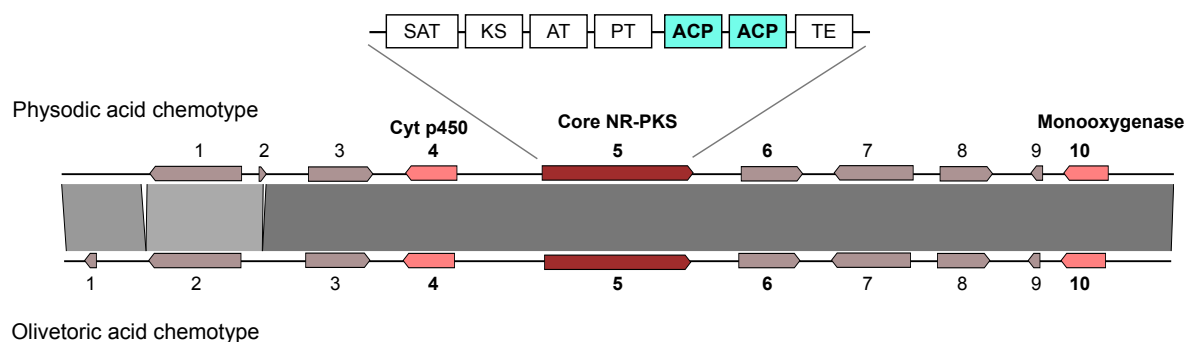
322 for: KS = keto-synthase, AT=acyltransferase, ACP=acyl carrier protein and KR=

323 ketoreductase. The PKS category is based on the phylogenetic placements of the *P. furfuracea*

324 NR-PKSs in the PKS groups from (Kim *et al.*, 2021). Cluster number refers to Supplementary

325 table S2 and the PKS number to the antiSMASH cluster, followed by the gene number.

326



327
 328 **Figure 3.** Synteny plot based on tBLASTn depicting conservation and synteny between the
 329 homologous putative cluster for the depsidone, physodic acid and the depside, olivetoric acid
 330 synthesis in *P. furfuracea*. Bolded numbers represent the *P. furfuracea* PKSs.

331
 332 We excluded the other seven NR-PKS clusters as possible candidates for
 333 olivetoric/physodic acid synthesis based on the following reasons: Cluster 1 has a cMT
 334 domain in the PKS, lacks *CytP450* and it *PKS* groups with group VII PKSs associated with
 335 the synthesis of azaphilones, monascorubrin related compounds. The *PKS* of cluster 2 lacks a
 336 TE domain, and it groups with *PKS*s linked to anthraquinone biosynthesis (group VII).
 337 *Cytochrome P450* is not present in this cluster. In cluster 3 *CytP450* is absent and the *PKS*
 338 groups with PKSs associated with melanin synthesis (group V). Cluster 5 contains a *R-PKS*
 339 and a *NR-PKS* next to each other divergently transcribed from the same region, suggesting
 340 common regulation and no connection to olivetoric acid synthesis. This cluster does contain a
 341 *CytP450*, but also an *O*-methyltransferase (*OMT*) which is not required for
 342 olivetoric/physodic synthesis. Cluster 6 contains a *CytP450*, but the *PKS* has a cMT domain
 343 and groups phylogenetically with atranorin-synthesizing PKSs (group IX). This is the most
 344 likely cluster involved in the synthesis of atranorin (see next paragraph). Clusters 7 and 8 lack
 345 *CytP450*, contain an *OMT*, and the PKSs group with melanin synthesizing PKSs (group II).
 346

347 **A putative atranorin cluster is present in *P. furfuracea***

348 Apart from the orcinol-derived olivetoric- and physodic acid, atranorin (a β -orcinol depside)
 349 is a common secondary metabolite produced by both chemotypes of *P. furfuracea*. Recently,
 350 the atranorin cluster from *Cladonia rangiferina* was characterized and heterologously
 351 expressed (Kim *et al.*, 2021). The atranorin PKS, PKS23, belongs to group IX (Fig. 2). The
 352 putative atranorin PKS is expected to have the following domains: SAT-KS-AT-PT-ACP-

353 cMT-TE. In our study, PKS Pfur9-3_011125 has this domain architecture and groups
354 phylogenetically with the atranorin cluster of *Cladonia rangiferina*. This cluster is present in
355 both chemotypes and has a gene composition similar to the atr1 cluster of *C. rangiferina*, i.e.,
356 it has a *CytP450*, an *OMT* and a transporter gene. We propose that this cluster is most likely
357 the atranorin cluster of *P. furfuracea*.

358

359 **The two genes for a metabolite FAS are present in *P. furfuracea***

360 *Aspergillus nidulans* has a metabolite FAS with properties similar to those expected for the
361 unexplored lichen metabolite FASs. The *A. nidulans* FAS comprises two subunits, HexA and
362 HexB, which produce and deliver to the aflatoxin NR-PKS the hexanoyl starter for
363 norsolorinic acid, the first metabolite in the pathway (Brown *et al.*, 1996; Watanabe and
364 Townsend, 2002). We used the *Cladonia grayi* homologs of *HexA* and *HexB* (DalGrande *et*
365 *al.*, in preparation) to search for the corresponding genes in *P. furfuracea*. We found one
366 5619-bp HexA homolog and one 6285-bp HexB homolog. Like in *A. nidulans* and *C. grayi*,
367 in both chemotypes of *P. furfuracea* these genes are adjacent and divergently transcribed from
368 the same control region (genes FUN_005930 and FUN_005931 in the olivetoric acid
369 chemotype; genes FUN_004275 and FUN_004276 in the physodic acid chemotype). These
370 FAS subunit genes are not linked to the olivetoric/physodic cluster.

371

372 **Transcription of the olivetoric/physodic BGC and of *HexA* and *HexB***

373 We checked the transcription of the genes of interest (genes of cluster 4 and *HexA*
374 and *HexB*) in lichen thalli of both chemotypes. In general, average transcription across the
375 genome was lower in the olivetoric than in the physodic chemotype. This was reflected in the
376 clusters as well. Transcriptome data suggest that in cluster 4, three genes out of 10, namely
377 the *Pfur33-1_006185*, the *CytP450* and *gene6* (coding for an unknown protein) were
378 transcriptionally active. We inferred the relative transcription activity by comparing the
379 number of transcriptome raw reads (normalized by counts per million) that aligned to the
380 respective gene (Table 4). Relative to the physodic acid (depsidone) chemotype, in the
381 olivetoric acid (depside) chemotype all genes of cluster4 showed low transcription activity,
382 although as compared to the other genes in the cluster the same three genes, *NR-PKS*,
383 *CytP450* and *gene6* showed higher transcription activity. *HexA* and *HexB* were transcribed in
384 both chemotypes. The number of read counts however, was higher in the physodic acid
385 chemotype than in the olivetoric acid chemotype. This parallels the behavior of the cluster 4
386 genes (Table 4).

387

Table 4. Read count of the genes of cluster 4 and *P. furfuracea* homologs of HexA and HexB of the olivetoric- and physodic acid chemotype.

Gene	gene identity	gene length	physodic aid chemotype		olivetoric acid chemotype	
			# raw reads aligned	reads (normalized by CPM)	# raw reads aligned	reads (normalized by CPM)
cluster 4 gene1	unidentified	3822	38	0	no hits	-
cluster 4 gene2	unidentified	306	no hits	-	1	0
cluster 4 gene3	unidentified	2685	no hits	-	19	0
cluster 4 gene4	cyt p450	2139	8677	117.29	1222	17.4
cluster 4 gene5	NR-PKS	6294	111226	511	1265	6.33
cluster 4 gene6	unidentified	2550	13690	155.25	6304	75.38
cluster 4 gene7	unidentified	3284	1035	9.11	118	1.125
cluster 4 gene8	unidentified	2191	936	12.35	205	1.98
cluster 4 gene9	unidentified	492	no hits	-	12	0.73
cluster 4 gene10	monooxygenase	1851	46	0.71	19	0.307
FAS	FAS-A	5619	3143	16.17	944	5.12
FAS	FAS-B	6285	1971	9.06	911	4.42

388

389 **Table 4.** Read count of the genes of cluster 4 and *P. furfuracea* homologs of *HexA* and *HexB*
390 of the olivetoric- and physodic acid chemotype. Transcriptome raw reads and normalized read
391 counts (by number of reads and gene length (RPKM approach)) aligned to the ten genes of the
392 candidate physodic and olivetoric acid cluster. Genes in bold (gene 4, 5, and 6) are the ones
393 with highest number of read counts.

394

395 Discussion

396 In this study we describe the putative BGCs for olivetoric- and physodic acid synthesis in the
397 lichen-forming fungus *P. furfuracea* from high quality long-read genome assemblies of the
398 two chemotypes. Furthermore, we identify the *HexA* and *HexB* homologs in *P. furfuracea*,
399 likely to deliver the starters to the orcinol compound PKSs. Combining our findings with
400 those of Kealey et al. (2021) and of other literature data, we propose an outline for the origin
401 of the starter unit, chemotype variation, and synthesis of orcinol depsides and depsidones in
402 lichens.

403

404 **True intraspecific variation underlies differences in BGCs between chemotypes**

405 While most (51) BGCs were present in both chemotypes, six BGCs were present only in one
406 chemotype (Supplementary info S2). In principle, the differences might be attributed to i)
407 random variation in sequencing depth, ii) contamination by another fungus, and iii) true
408 intraspecific variation. Random sequencing depth variation can be excluded, because we
409 detected no reads of the missing BGC in the raw data. It is very unlikely that large genomic
410 regions, such as entire BGCs, would be missed due to uneven coverage. Contamination from
411 reads of minority fungal genomes (e.g. from lichenicolous fungi) can also be excluded, as we
412 did not detect any coverage variation with underrepresented sequences compared to those
413 from the main mycobiont. Furthermore, the clusters detected only in the physodic acid
414 chemotype were also absent in the previously sequenced genome of *Pseudevernia furfuracea*,
415 which was from the olivetoric acid chemotype (Meiser *et al.*, 2017). True intraspecific
416 variation is therefore the most likely cause of the observed differences in BGC content
417 between chemotypes.

418 Intraspecies variations in BGCs have been reported for plants, bacteria, and fungi,
419 and have been linked to ecological adaptation (Moore *et al.*, 2014; Zhu *et al.*, 2016; Thynne *et al.*,
420 *et al.*, 2019; Drott *et al.*, 2020). For instance, the number of BGCs may vary between
421 populations inhabiting different climatic conditions (Drott *et al.*, 2020; Singh *et al.*, 2021). In
422 fact, fungal BGCs are suggested to be hotspots of gene gain/loss and duplication (Wisecaver
423 *et al.*, 2014; Lind *et al.*, 2017; Rokas *et al.*, 2018). Different strains of a single species can
424 contain up to 15 strain-specific clusters (Vicente *et al.*, 2018). The presence of unique BGCs
425 suggests that each chemotype has a specialized metabolite potential based on genetic
426 differences. Genome sequences of only two individuals are not likely to capture the
427 pangenomic variation of BGCs within *P. furfuracea*. BGC variation among individuals of a
428 species appears to be a common phenomenon and therefore a single individual may not
429 represent the entire biosynthetic potential of a species (Susca *et al.*, 2016; Villani *et al.*, 2019;
430 Singh *et al.*, 2021). However, intraspecific biosynthetic variation can also arise when the
431 BGCs involved are present in all individuals, as exemplified by the BGC likely responsible
432 for the synthesis of olivetoric acid and physodic acid (see below).

433 Our results suggest that differences in presence/absence of BGCs are not linked to
434 differences between chemotypes. Although here are many cluster differences between
435 chemotypes, these differences do not affect the chemistry of the two chemotypes. Instead, the
436 chemotypic differences appear to be because of the divergent regulation of the same cluster
437 present in both chemotypes.

438

439 **The same candidate BGC is linked to depside and depsidone biosynthesis**

440 The cluster we identified as the likely BGC linked to olivetoric/physodic biosynthesis has
441 identical gene content in both chemotypes (Fig. 3), prompting the question of how one
442 chemotype produces largely the depside olivetoric acid and the other largely the depsidone
443 physodic acid. The likely BGC includes a *NR-PKS* and a *CytP450*, the two essential
444 requirements for depside and depsidone synthesis (Armaleo et al. 2011; Kealey et al. 2021).
445 These are also two of the three most highly transcribed genes in the cluster (Table 4). The
446 function of the other genes, which are unidentified and mostly transcriptionally silent, with
447 regard to olivetoric/physodic acid synthesis is unknown (Table 4). Theoretically, differential
448 transcription of *CytP450* could explain the difference between chemotypes: while the *PKS*
449 should be expressed in both chemotypes, repression of the *CytP450* gene in the olivetoric
450 chemotype would prevent the depside to depsidone transition, whereas expression of the
451 *CytP450* in the physodic chemotype would allow depsidone synthesis. However, the
452 transcriptome data (Table 4) shows that *CytP450* is transcribed in both chemotypes, probably
453 because averaging reads from thalli comprising different developmental and physiological
454 stages cannot reflect subtle developmental transitions occurring at different times and
455 locations. In fact each chemotype may occasionally produce both, the depside and the
456 depsidone, depending upon the regulatory and other factors, but one of the two compounds
457 remains below the level of detection. There are reports of occasional thalli of *P. furfuracea*
458 containing both, physodic acid and olivetoric acid (Culberson *et al.*, 1977).

459 While at a cellular scale differential transcription is decisive in determining
460 phenotypes in fungi, secondary metabolite synthesis is a complex, multi-step process
461 involving various genetic, epigenetic and environmental factors that together determine the
462 spatio-temporal secondary metabolite profile of an organism (Fox and Howlett, 2008;
463 Macheleidt *et al.*, 2016; Keller, 2019). Often, the same BGC can be differentially regulated at
464 the intraspecies level epigenetically, posttranscriptionally or posttranslationally, to produce
465 different compounds (Yin and Keller, 2011; Patra *et al.*, 2013; Collemare and Seidl, 2019;
466 Drott *et al.*, 2020). For instance, the aspyridone cluster in *Aspergillus nidulans* can produce up
467 to eight different compounds depending on the combination of genes activated (Wasil *et al.*,
468 2013). Although our findings cannot explain which aspects of this complexity differentiate the
469 chemotypes of *P. furfuracea*, they clearly indicate that differential regulation of the same
470 BGC is involved. Our study shows that biosynthetic capabilities of organisms may vary

471 within a species and highlights the importance of exploring the biosynthetic potential of
472 organisms at the intraspecies level.

473

474 **A metabolite fatty acyl synthase is the likely provider of the hexanoyl starter for**
475 **olivetoric acid synthesis**

476 We found the homologs of *HexA* and *HexB* in *P. furfuracea*. As in *Aspergillus nidulans* and
477 *Cladonia grayi* these genes are located next to each other and in divergent orientation,
478 suggesting that they are co-regulated by the same promoter. HexA and HexB refer
479 respectively to the α and β subunits of the hexanoate synthase in *A. nidulans*. The FAS
480 domains ACP, KR and KS are present in the α -chain and AT, ER, DH and malonyl-ACP
481 transferase (MPT) in the β -chain (Jenni *et al.*, 2006). HexA/B provides the hexanoyl starter to
482 the PKS synthesizing the norsolorinic acid precursor of aflatoxin in *Aspergillus*. We propose
483 that the HexA/B homolog in *P. furfuracea* delivers hexanoyl starters to initiate both rings of
484 olivetoric acid, although the A-ring side chain ends up being two-carbons longer than the B-
485 ring chain (Fig. 4), as described in the next section.

486 The PKS of cluster4 we identified in this study as most likely associated with
487 olivetoric/physodic acid biosynthesis, is the same as *Pfur33-1_006185* that was
488 heterologously expressed in yeast by Kealey *et al.* (2021). Interestingly, expression of this
489 PKS in the heterologous host yielded lecanoric acid (Kealey *et al.* 2021), a compound never
490 reported from thalli of *P. furfuracea*. Lecanoric acid and olivetoric acid differ in their starter
491 side chains: both rings of olivetoric acid are started by an hexanoyl chain (Fig. 4) whereas
492 both rings in lecanoric acid are started by the two-carbon chain from Acetyl CoA. This
493 indicates that a PKS specific for olivetoric acid in *P. furfuracea* accepts Acetyl CoA as starter
494 in yeast while it never does so in the lichen where it only accepts hexanoyl chains. Moreover,
495 the heterologously expressed PKS continued to prefer acetyl CoA in yeast and produce
496 lecanoric acid even when hexanoyl CoA was provided (Kealey *et al.* 2021). A likely solution
497 to these apparent contradictions is that the “default” setting for the olivetoric PKS and perhaps
498 for other orcinol depside PKSs is to accept free acetyl CoA as starter, but not free acyl CoAs
499 with longer chains. This default setting is revealed only in the absence of a dedicated
500 metabolite FAS like the one we identified in the lichen. Yeast has no metabolite FAS genes.
501 The task of the metabolite FAS is to transfer directly to the PKS, through specific binding of
502 the two proteins, the hexanoyl chain from the FAS ACP to the PKS ACP, with no free acyl
503 CoA intermediate. That would explain why in yeast the olivetoric PKS would not use free
504 hexanoyl CoA. Such an acyl-transfer mechanism is identical to what has been proposed for

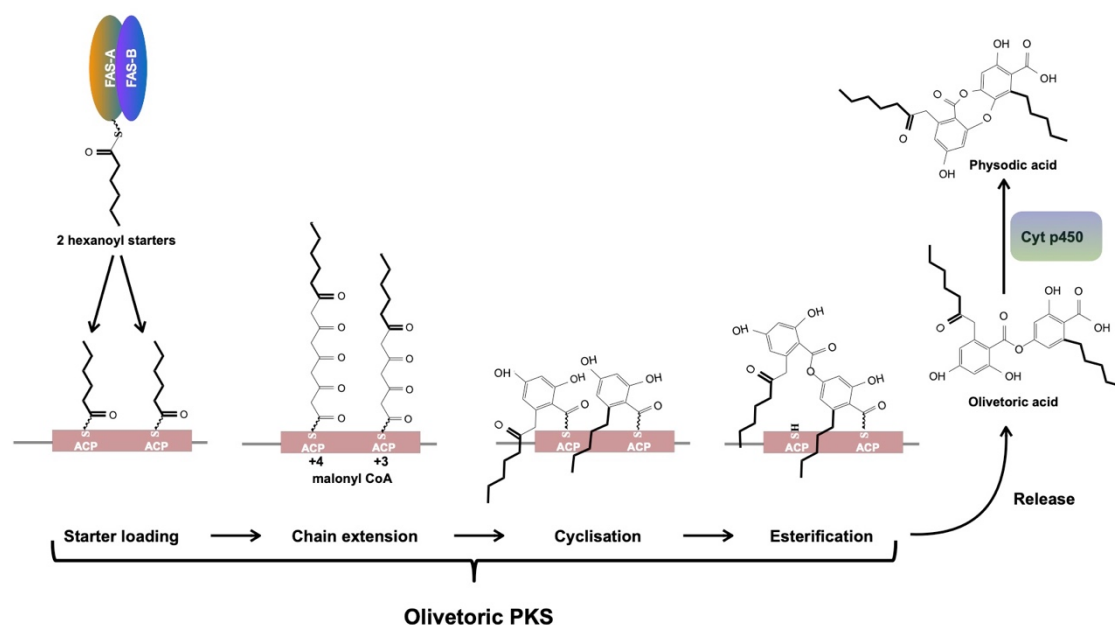
505 hexanoyl transfer between the HexA-HexB FAS in *A. nidulans* and the norsolorinic acid PKS
506 (Watanabe *et al.*, 1996; Watanabe and Townsend, 2002). Our identification in *P. furfuracea*
507 of an expressed (Table 4) close homolog of the *A. nidulans* HexA-HexB FAS provides strong
508 support for our proposed scenario. An important corollary of this scenario is that the side
509 chain specificity in lichen orcinol compounds is not controlled exclusively by PKSs but likely
510 results from specific protein-protein interactions between each PKS and a dedicated
511 metabolite FAS which synthesizes and delivers the appropriate acyl-ACP starter directly to
512 the PKS.

513

514 **An updated scheme of orcinol depside and depsidone synthesis**

515 We combine here our results with those of Watanabe and Townsend (2002) Armaleo *et al*
516 (2011), Feng *et al.* (2019), Lünne *et al.*, (2020), and Kealey *et al.*, (2021), to provide an
517 updated scheme of orcinol depside and depsidone synthesis, using as example the synthesis of
518 olivetoric and physodic acid. We limit our description to orcinol lichen compounds as we do
519 not yet know how many of the same rules apply to β -orcinol depsides and depsidones. Orcinol
520 and β -orcinol PKSs are separated by a deep evolutionary gulf (Fig. 2) and the biological
521 differences between these two groups of lichen compounds are not well understood.

522



523

524 **Figure 4.** Putative scheme for depside/depsidone synthesis and function of FASs.

525

526 The scheme is depicted in Figure 4. Unless acetyl CoA provides the starter, as is
527 the case for lecanoric acid and other orcinol compounds with methyl groups as side chains, a
528 dedicated HexA/B FAS is needed to provide an acyl-ACP as starter to the *PKS*. In the case of
529 olivetoric acid, hexanoyl-ACP is the starter for both rings and is transferred within the two
530 proteins bound to each other from the FAS-ACP to the *PKS* ACPs (Fig. 4). The symmetric
531 addition of starters is not the rule, as many orcinol compounds use different acyl chain starters
532 for the two rings. Polyketide extension involves a minimum of three malonyl CoA additions,
533 but can involve four. The *PKS* then cyclizes both polyketide chains to orcinol rings, esterifies
534 the carboxyl of the A ring with the 4' OH of the B ring and finally releases the depside by
535 hydrolysis of the B ring thioester. The rings produced after four malonyl additions commonly
536 have side chains with a β -keto group derived from the carbonyl oxygen of the hexanoyl starter
537 (Fig. 4), as seen on the A ring side chain of olivetoric and physodic acid. If the released
538 depside is to be turned into a depsidone, a dedicated cytochrome P450 adds an ether bond,
539 oxidatively coupling the C2 OH of the A ring to the 5' C of the B ring.

540

541 **Conclusions**

542 Our study contributes to the understanding of natural product synthesis in lichenized fungi in
543 several ways. We identified the BGCs of the two *P. furfuracea* chemotypes and highlighted
544 the putative cluster linked to physodic- and olivetoric acid biosynthesis. Additionally, we
545 characterized the *P. furfuracea* homologs of *HexA/HexB*, the first FASs from lichen-forming
546 fungi putatively involved in metabolite synthesis. Taken together, our results show that the
547 same BGC has the potential to produce different compounds and suggests that intraspecific
548 variation in the regulation of metabolite synthesis adds to the biosynthetic diversity and
549 potential of organisms despite similar BGC content. Our study helped clarify some of the
550 components determining chemotype variability in lichens and, in combination with other data,
551 has allowed us to devise the most detailed scheme to date for the synthesis of orcinol depsides
552 and depsidones. However, although the scheme combines the available evidence in a way
553 consistent with the known molecular biology and biochemistry of these compounds, a number
554 of details remain hypothetical and need experimental confirmation.

555

556

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561 have no conflict of interest.

562

563 **Supporting information**

564 **Table S1.** Voucher information of the samples used for the study

565

566 **Table S2.** Details of the estimated biosynthetic gene clusters in the two chemotypes of *P.*
567 *furfuracea*. PKS number (column 2) refers to the most closely related PKS in the maximum
568 likelihood tree presented in Figure 2. Region (column 3) refers to the cluster number and
569 regions in the antiSMASH output.

570

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