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NATURAL PRODUCTS - MINI REVIEW

A comprehensive catalogue of polyketide synthase gene clusters in lichenizing fungi

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Abstract

Lichens are fungi that form symbiotic partnerships with algae. Although lichens produce diverse polyketides, difficulties in establishing and maintaining lichen cultures have prohibited detailed studies of their biosynthetic pathways. Creative, albeit non-definitive, methods have been developed to assign function to biosynthetic gene clusters in lieu of techniques such as gene knockout and heterologous expressions that are commonly applied to easily cultivatable organisms. We review a total of 81 completely sequenced polyketide synthase (PKS) genes from lichenizing fungi, comprising to our best efforts all complete and reported PKS genes in lichenizing fungi to date. This review provides an overview of the approaches used to locate and sequence PKS genes in lichen genomes, current approaches to assign function to lichen PKS gene clusters, and what polyketides are proposed to be biosynthesized by these PKS. We conclude with remarks on prospects for genomics-based natural products discovery in lichens. We hope that this review will serve as a guide to ongoing research efforts on polyketide biosynthesis in lichenizing fungi.

Keywords Natural products · Gene clusters · Lichens · Genome sequencing · Metabolite prediction · Polyketides

Introduction

Lichens, traditionally described as a symbiosis between fungi (mycobiont) and algae (photobiont), are miniature ecosystems of fungi cohabitating with diverse species of algae, yeast, and bacteria [14, 46, 72, 95]. By convention, the taxonomic name of the mycobiont is also the name of the symbiotic complex. The name of a lichen, therefore, refers to the fungus, and the fungus may exist either as part of a symbiosis in nature or as an isolated sub-culture in laboratory. Lichens comprise 20% of all fungi and can be found in every terrestrial habitat [18, 68]. Lichens produce more than 1000 secondary metabolites, many of which are used as additives in commercial products or are being investigated as pharmaceuticals [25, 56, 73, 92, 124] (Fig. 1). Although the photobiont is known to produce a few natural products [60, 61], the mycobiont is thought to be responsible for the majority of secondary metabolites produced by the symbiotic complex. Polyketides are the most studied class of secondary metabolites produced by lichens. Lichen polyketides typically comprise aromatic polyphenols as well as cyclic and linear aliphatic compounds (Fig. 1). Polyketides are produced by polyketide synthases (PKS), multidomain enzymes that condense acetyl- and malonyl-CoA [96, 116]. The initial products of PKS can then be 'tailored' by accessory enzymes yielding complex structures. Well-studied examples of polyketides among non-lichenizing fungi include aflatoxin, patulin, and lovastatin [26, 85, 87].

Despite the rich chemical diversity of lichens, few biosynthetic studies are available. This is in large part because of the challenges of establishing and growing lichen cultures within the laboratory. To study secondary metabolite biosynthesis, it is often necessary to isolate and maintain pure cultures of the fungal partner. Separating lichen partners is challenging because this is an obligate symbiosis [45]. The lichen lifespan can extend to thousands of years and a rate of biomass accumulation of 1-cm-diameter growth per year is typical for many species [45]. This slow rate of growth of isolated fungal partners greatly complicates the performance

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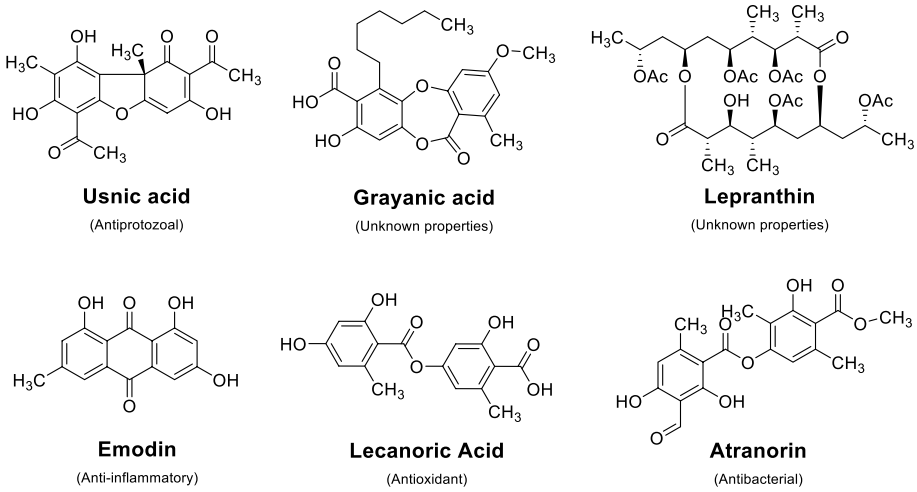

Fig. 1 Selected lichen polyketides: Usnic acid [10], grayanic acid [11], lepranthin [84], emodin [90], lecanoric acid [65], atranorin [97]

of tasks such as time-course induction experiments of natural product biosynthesis or the isolation of DNA from sub-cultured mycobionts for genome sequencing. The chemical profile of lichens appears to be sensitive to environmental conditions as it is often observed that the chemical profile of lichens in natural habitats is different from cultivated thallus as well as mycobiont sub-cultures grown in the laboratory [45].

Recently, the goal in studying natural product biosynthesis is to identify the biosynthetic gene cluster that is responsible for the production of a specific natural product. Gain- or loss-of-function experiments can be used to link genes to molecules to determine the nature and sequence of chemical transformations within a biosynthetic pathway. Techniques used include gene knockout, RNA interference, and functional heterologous expression [6, 7, 41, 43, 50]. In non-lichenizing fungi and bacteria, these techniques have been used to determine the genetic origins of dozens of secondary metabolites [62, 67]. In contrast, no lichen secondary metabolite has been definitively linked to a gene cluster through gene knockout, RNA interference, or functional heterologous expression experiments. Definitive assignment of function of lichen biosynthetic gene clusters remains elusive.

Creative methods of studying natural product biosynthesis have been developed to overcome the challenges of working with lichen cultures. In this review, we summarize the current approaches to locate PKS gene clusters within lichen genomes. The methods used to assign putative biosynthetic functions to the encoded PKS are also reviewed. This article may also serve as a catalogue of all completely sequenced and GenBank-deposited PKS genes known to date from lichenizing fungi.

General findings

A total of 81 PKS genes have been compiled from 13 species of lichenizing fungi. This list includes 38 type I non-reducing PKS (Table 1), 41 type I reducing PKS (Table 2), and 2 type III PKS (Table 3). The tables are arranged according to the architecture of individual domains in the gene coding for the PKS, followed by species of origin. Information on putative post-PKS accessory tailoring genes that appear to be associated with these PKS genes is available for 37 of 81 entries. These accessory genes are listed alongside the catalogued PKS in Tables 1, 2, 3. The most common accessory genes encode cytochrome p450 oxidases, FAD-dependent oxidases, dehydrogenases, and *O*-methyltransferases.

Type III PKS do not have distinct catalytic domains but prime and extend polyketides to a length determined by the size of the catalytic pocket [64, 91]. Two complete type III PKS genes have been reported, both in *Cladonia uncialis*, and both appearing to encode chalcone synthases (Table 3). These assignments are based on BLAST consensus similarity with other chalcone synthases deposited in GenBank [15]. Many other type III PKS likely await characterization because PCR amplification experiments have demonstrated that type III PKS are found in many species of lichen; however, complete gene sequences were not reported [66]. As we focused exclusively on complete PKS genes, these partially amplified PKS genes were not included in the review. Information on these partial genes can be found elsewhere [66].

Several draft genomes of lichen mycobionts have been reported that have provided additional insight into the ubiquity of PKS genes throughout lichenizing fungi [79–83, 112]. In these reports, domain search tools (e.g., InterPro

Table 1 Type I non-reducing polyketide synthase genes in lichenizing fungi

Entry	Name	Accession	Species	Architecture	Putative tailoring enzymes	Detection method	Putative function	Prediction method	Ref(s)
1	*Cgrpks15*	HQ823621	*Cladonia grayi*	SAT-KS-AT-PT-ACP-TE	–	cDNA—genome assembly	–	–	[11]
2	*Cmapks1*	JQ340775	*Cladonia macilenta*	SAT-KS-AT-PT-ACP-TE	–	gDNA—library screening	Melanin/orsellinic acid-related	Phylogenetics	[58]
3	*Cu-nr-pks-4*	MG777492	*Cladonia uncialis*	SAT-KS-AT-PT-ACP-TE	p450	Genome sequencing	–	–	[15, 16]
4	*Cu-nr-pks-5*	MG777493	*Cladonia uncialis*	SAT-KS-AT-PT-ACP-TE	De[CO2]	Genome sequencing	–	–	[15, 16]
5	*Cu-nr-pks-14*	MG777502	*Cladonia uncialis*	SAT-KS-AT-PT-ACP-TE	MT, Hyd	Genome sequencing	–	–	[15, 16]
6	*Ulpks6*	JX232188	*Usnea longissima*	SAT-KS-AT-PT-ACP-TE	–	gDNA—library screening	Orsellinic acid-related	Phylogenetics	[113]
7	*Cgrpks13*	HQ823619	*Cladonia grayi*	SAT-KS-AT-PT-ACP-ACP-TE	–	cDNA—genome assembly	–	–	[11]
8	*Cgrpks14*	HQ823620	*Cladonia grayi*	SAT-KS-AT-PT-ACP-ACP-TE	–	cDNA—genome assembly	–	–	[11]
9	*Cgrpks16*	GU930713	*Cladonia grayi*	SAT-KS-AT-PT-ACP-ACP-TE	p450, MT	cDNA—genome assembly	Grayanic acid	Transcription profiling	[11]
10	*Cu-nr-pks-1*	MG777489	*Cladonia uncialis*	SAT-KS-AT-PT-ACP-ACP-TE	MT, SDR, De[H2O], FAD, AldRed, FAD	Genome sequencing	–	–	[15, 16]
11	*Cu-nr-pks-6*	MG777494	*Cladonia uncialis*	SAT-KS-AT-PT-ACP-ACP-TE	SDR, De[H2O], Mono, p450	Genome sequencing	–	–	[15, 16]
12	*Cu-nr-pks-7*	MG777495	*Cladonia uncialis*	SAT-KS-AT-PT-ACP-ACP-TE	p450, MT	Genome sequencing	Grayanic acid	Homology mapping	[15, 16]
13	*Cu-nr-pks-8*	MG777496	*Cladonia uncialis*	SAT-KS-AT-PT-ACP-ACP-TE	Mono, DH-KR, Halo, MT	cDNA—RACE	Terrein-related	Homology mapping	[2, 15]
14	*Cu-nr-pks-9*	MG777497	*Cladonia uncialis*	SAT-KS-AT-PT-ACP-ACP-TE	Hyd, OR	Genome sequencing	–	–	[15, 16]
15	*Cu-nr-pks-10*	MG777498	*Cladonia uncialis*	SAT-KS-AT-PT-ACP-ACP-TE	Hyd, MT, p450, Mono, Mono, SDR, ACP-R	Genome sequencing	Fusarubin-related	Homology mapping	[15, 16]
16	*Dnpks1*	EU872212	*Dirinaria applanata*	SAT-KS-AT-PT-ACP-ACP-TE	–	gDNA—library screening	Melanin/anthraquinone-related	Phylogenetics	[106]
17	*Eppks3*	ERF69996	*Endocarpon pusillum*	SAT-KS-AT-PT-ACP-ACP-TE	–	Genome sequencing	–	–	[110]
18	*Eppks4*	ERF77221	*Endocarpon pusillum*	SAT-KS-AT-PT-ACP-ACP-TE	–	Genome sequencing	–	–	[110]

Table 1 (continued)

Entry	Name	Accession	Species	Architecture	Putative tailoring enzymes	Detection method	Putative function	Prediction method	Ref(s)
19	*Pmpks4*	HM180407	*Peltigera membranacea*	SAT-KS-AT-PT-ACP-ACP-TE	–	Genome sequencing	–	–	[61]
20	*Pmpks6*	HM180409	*Peltigera membranacea*	SAT-KS-AT-PT-ACP-ACP-TE	–	Genome sequencing	–	–	[61]
21	*Ulpks1*	JN408682	*Usnea longissima*	SAT-KS-AT-PT-ACP-ACP-TE	Red, Unk, Unk	gDNA—library screening	Melanin/orsellinic acid-related	Phylogenetics	[109]
22	*Ulpks2*	JX232185	*Usnea longissima*	SAT-KS-AT-PT-ACP-ACP-TE	–	gDNA—library screening	Orsellinic acid-related	Phylogenetics	[113]
23	*Xsmpks1*	KJ501919	*Xanthoparmelia substrigosa*	SAT-KS-AT-PT-ACP-ACP-TE	–	cDNA—RACE	–	–	[51]
24	*Xepks1*	DQ660910	*Xanthoria elegans*	SAT-KS-AT-PT-ACP-ACP-TE	–	cDNA—RACE	Anthraquinone-related	Phylogenetics	[23]
25	*Cu-nr-pks-11*	MG777499	*Cladonia uncialis*	SAT-KS-AT-PT-ACP-MT-TE	p450, GMC, p450, SDR, Lactam, MT, GMC, SDR	Genome sequencing	Mycophenolic acid-related	Homology mapping	[15, 16]
26	*Ulpks4*	JX232186	*Usnea longissima*	SAT-KS-AT-PT-ACP-MT-TE	–	gDNA—library screening	Methylorsellinic acid-related	Phylogenetics	[113]
27	*Cu-nr-pks-2*	MG777490	*Cladonia uncialis*	SAT-KS-AT-PT-ACP-MT-CYC	p450	Genome sequencing	Usnic acid	Gene surveying	[3]
28	*Cu-nr-pks-12*	MG777500	*Cladonia uncialis*	SAT-KS-AT-PT-ACP-MT-CYC	GNAT, p450	Genome sequencing	–	–	[15, 16]
29	*Cgrpks2*	GU930714	*Cladonia grayi*	SAT-KS-AT-PT-ACP-MT-CYC	–	cDNA—genome assembly	–	–	[11]
30	*Cgrpks1*	HQ823618	*Cladonia grayi*	SAT-KS-AT-PT-ACP-ACP-MT-CYC	–	cDNA—genome assembly	–	–	[11]
31	*Eppks1*	ERF73753	*Endocarpon pusillum*	SAT-KS-AT-PT-ACP-R	–	Genome sequencing	–	–	[110]
32	*Hypopks1*	JX067626	*Hypogymnia physodes*	SAT-KS-AT-PT-ACP-MT-R	–	gDNA—library screening	Methylorcinolaldehyde-related	Phylogenetics	[121]
33	*Hypopks2*	JX067627	*Hypogymnia physodes*	SAT-KS-AT-PT-ACP-MT-R	–	gDNA—library screening	Methylorcinolaldehyde-related	Phylogenetics	[121]
34	*Cu-nr-pks-3*	MG777491	*Cladonia uncialis*	SAT-KS-AT-PT-ACP-ACP-MT-R	De[H], FAD, Mono, FAD, AT, OR, OR	Genome sequencing	Azaphilone-related	Homology mapping	[15, 16]
35	*Eppks2*	ERF73358	*Endocarpon pusillum*	SAT-KS-AT-PT-ACP-ACP-MT-R	–	Genome sequencing	–	–	[110]
36	*Cu-nr-pks-13*	MG777501	*Cladonia uncialis*	SAT-KS-AT-PT-ACP	Mono, Lactam, MT	Genome sequencing	Pestheic acid-related	Homology mapping	[15, 16]

Table 1 (continued)

Entry	Name	Accession	Species	Architecture	Putative tailoring enzymes	Detection method	Putative function	Prediction method	Ref(s)
37	*Ulpks5*	JX232187	*Usnea longissima*	SAT-KS-AT-PT-ACP	Lactam, De[H2O]	gDNA—library screening	Anthraquinone-related	Phylogenetics	[115]
38	*Xsepks1*	ABS58604	*Xanthoparmelia semiviridis*	SAT-KS-AT-PT-ACP-MT	–	gDNA—library screening	Methylorsellinic acid-related	Phylogenetics	[29]

PKS domain abbreviations: starter unit-acyl-carrier protein transacylase (SAT)|Ketosynthase (KS)|Acyltransferase (AT)|Product template domain (PT)|Acyl carrier protein (ACP)|C-methyltransferase (MT)|Thioesterase (TE)|Claisen cyclase (CYC)|Reductase (R) Accessory gene abbreviations: No information available (–)|Cytochrome p450 oxidase (p450)|O-methyltransferase (MT)|Short-chain dehydrogenase/reductase (SDR)|Dehydratase (De[H2O])|Flavin adenine dinucleotide-dependent oxidase (FAD)|Aldehyde reductase (AldRed)|Dehydrogenase (De[H])|Monooxygenase (Mono)|Acetyltransferase (AT)|Oxidoreductase (OR)|Decarboxylase (De[CO2])|Trans-PKS dehydratase and enoyl-reductase domains (DH-KR)|Halogenase (Halo)|Hydratase (Hyd)|Trans-PKS acyl carrier protein and reductase domains (ACP-R)|Glucose-methanol choline oxidoreductase (GMC), GCN5-related N-acetyltransferase (GNAT)|Unknown function (Unk)|Beta-lactamase (Lactam)

[57]) were used to provide evidence for the existence of PKS genes within these fungal genomes. However, these reports did not include a detailed annotation of these PKS genes within a genomic DNA sequence nor were nucleotide accession numbers provided for individual PKS genes. For this reason, we were unable to include these PKS genes within our tabulated results.

PKS domain architecture

PKS are generally classified as either reducing or non-reducing based on the presence of catalytic domains that determine the extent of carbon reduction. Fungal non-reducing PKS minimally require a starter unit-acyl-carrier protein transacylase (SAT), a ketosynthase (KS), an acyltransferase (AT), an acyl-carrier protein (ACP), a product template domain (PT), and a terminal domain comprising either of thioestase (TE), Claisen cyclase (CYC), or reductase (R) [96, 116]. The presence of a *C*-methyltransferase domain (MT) is optional. The SAT and PT domains of some PKS compiled here were not annotated in the original report, presumably because the discovery and functional characterization of these domains have occurred relatively recently [31, 32]. We, therefore, updated the reported architecture of some of these PKS using BLAST [8] and AntiSMASH (v. 4.0) [20]. Non-reducing PKS typically form aromatic polyphenols. Fungal-reducing PKS lack SAT, PT, and terminal domains, but reduce β-carbons using ketoreductase (KR), dehydratase (DH), and enoyl-reductase (ER) domains [28, 30, 93]. Reducing PKS can form reduced aromatic rings such as 6-methylsalicylic acid as well as aliphatic rings such as macrolides and lactones. The AT domain of fungal PKS most commonly recruit malonyl-CoA to extend the PKS, though numerous exceptions have been reported [27]. No speculation can be provided on the type of extension unit recruited by the AT domain of each of the encoded PKS, with exception to speculations that could be provided based on the structure of the metabolites putatively associated with some of these PKS genes.

A total of 38 type I non-reducing PKS from lichenizing fungi are catalogued in Table 1. Examples of the minimalistic iterative PKS architecture are provided in entries 1–6 in Table 1. These genes contain only the SAT-KS-AT-PT-ACP-TE domains that are associated with the biosynthesis of polyphenols. Entries 7–24, 30, 34 and 35 all contain an additional ACP domain. Tandem ACP is more commonly found in fatty acid synthases (FAS) than PKS. Mutagenesis experiments suggest that the biological role of tandem ACP is to increase product turnover because both ACP are functionally equivalent and may operate in parallel [47, 59, 86]. How mechanistically this occurs remains unclear. Bioengineering FAS to possess more ACP can increase product turnover [52]. The PKS responsible for the biosynthesis of 1,3,6,8-tetrahydroxynaphthalene, sterigmatocystin, and albicidin are three examples of PKS from non-lichen organisms possessing two ACP domains [39, 55, 120]. We speculate that the role of double ACP domains observed in lichenizing fungi is to increase the catalytic turnover of the encoded PKS.

So-called '*trans*-PKS' lack one or more catalytic domains. In these cases, lost functionalities are provided by domain-like enzymes encoded near the PKS gene [53]. A *trans*-enoyl reductase participating in lovastatin biosynthesis is a well-characterized example of a *trans*-PKS from non-lichenizing fungi [9]. Two *trans*-PKS have been identified, both in *C. uncialis*. One DH-KR-like gene near *Cu-nr-pks*-8 (Entry 13) was proposed to be involved in 6-hydroxymellein biosynthesis (vide infra) [2]. An ACP-R-like gene without proposed function was observed near *Cu-nr-pks-10* (Entry 15) [15]. These putative *trans*-PKS await functional characterization.

Table 2 Type I reducing polyketide synthase genes in lichenizing Fungi

Entry	Name	Accession	Species	Architecture	Putative tailoring enzymes	Detection method	Putative function	Prediction method	Ref(s)
39	*Cmpks1*	HQ413098	*Cladonia metacorallifera*	KS-AT-DH-KR-ACP	LAAO, Mono, Unk	gDNA—library screening	Methylsalicylic acid-related	Phylogenetics	[63]
40	*Cu-r-pks-3*	MG777496	*Cladonia uncialis*	KS-AT-DH-KR-ACP	MT	Genome sequencing	–	–	[15, 16]
41	*Cu-r-pks-9*	MG777507	*Cladonia uncialis*	KS-AT-DH-KR-ACP	Oxy, De[H], p450, FAD, De[CO2], p450, OR, CE, SDR, Red, GMC	Genome sequencing	Patulin	Homology mapping	[15, 16]
42	*Eppks5*	ERF77015	*Endocarpon pusillum*	KS-AT-DH-KR-ACP	–	Genome sequencing	–	–	[110]
43	*Eppks14*	ERF69189	*Endocarpon pusillum*	KS-AT-DH-KR-ACP	–	Genome sequencing	–	–	[110]
44	*Cu-r-pks-12*	MG777510	*Cladonia uncialis*	KS-AT-DH-MT-KR-ACP	SDR	Genome sequencing	–	–	[15, 16]
45	*Cu-r-pks-4*	MG777497	*Cladonia uncialis*	KS-AT-DH-ER-KR-ACP	[None]	Genome sequencing	–	–	[15, 16]
46	*Cu-r-pks-6*	MG777504	*Cladonia uncialis*	KS-AT-DH-ER-KR-ACP	De[H], De[CO2], Hyd, AlaLig, De[H2O]	Genome sequencing	–	–	[15, 16]
47	*Cu-r-pks-10*	MG777508	*Cladonia uncialis*	KS-AT-DH-ER-KR-ACP	Hyd	Genome sequencing	–	–	[15, 16]
48	*Cu-r-pks-11*	MG777509	*Cladonia uncialis*	KS-AT-DH-ER-KR-ACP	[O], p450, [O], De[H], p450	Genome sequencing	–	–	[15, 16]
49	*Eppks11*	ERF75982	*Endocarpon pusillum*	KS-AT-DH-ER-KR-ACP	–	Genome sequencing	–	–	[110]
50	*Eppks12*	ERF73411	*Endocarpon pusillum*	KS-AT-DH-ER-KR-ACP	–	Genome sequencing	–	–	[110]
51	*Eppks13*	ERF68064	*Endocarpon pusillum*	KS-AT-DH-ER-KR-ACP	–	Genome sequencing	–	–	[110]
52	*Eppks15*	ERF76568	*Endocarpon pusillum*	KS-AT-DH-ER-KR-ACP	–	Genome sequencing	–	–	[110]
53	*Ulpks3*	HQ824546	*Usnea longissima*	KS-AT-DH-ER-KR-ACP	Hyd, Unk	gDNA—library screening	Resorcylic acid lactone-related	Phylogenetics	[108]
54	*Cu-r-pks-1*	MG777470	*Cladonia uncialis*	KS-AT-DH-MT-ER-KR-ACP	[None]	Genome sequencing	–	–	[15, 16]
55	*Cu-r-pks-2*	MG777488	*Cladonia uncialis*	KS-AT-DH-MT-ER-KR-ACP	p450, SDR, p450, p450	Genome sequencing	–	–	[15, 16]
56	*Cu-r-pks-5*	MG777503	*Cladonia uncialis*	KS-AT-DH-MT-ER-KR-ACP	OR, TE, Red	Genome sequencing	–	–	[15, 16]
57	*Cu-r-pks-7*	MG777505	*Cladonia uncialis*	KS-AT-DH-MT-ER-KR-ACP	MT, Hyd	Genome sequencing	–	–	[15, 16]
58	*Cu-r-pks-8*	MG777506	*Cladonia uncialis*	KS-AT-DH-MT-ER-KR-ACP	SDR	Genome sequencing	–	–	[15, 16]
59	*Cu-r-pks-13*	MG777511	*Cladonia uncialis*	KS-AT-DH-MT-ER-KR-ACP	De[CO2], De[H]	Genome sequencing	–	–	[15, 16]

Table 2 (continued)

Entry	Name	Accession	Species	Architecture	Putative tailoring enzymes	Detection method	Putative function	Prediction method	Ref(s)
60	*Cu-r-pks-14*	MG777512	*Cladonia uncialis*	KS-AT-DH-MT-ER-KR-ACP	AldDe[H], De[CO2]	Genome sequencing	–	–	[15, 16]
61	*Cu-r-pks-16*	MG777514	*Cladonia uncialis*	KS-AT-DH-MT-ER-KR-ACP	OR	Genome sequencing	–	–	[15, 16]
62	*Cu-r-pks-17*	MG777515	*Cladonia uncialis*	KS-AT-DH-MT-ER-KR-ACP	[None]	Genome sequencing	–	–	[15, 16]
63	*Cu-r-pks-18*	MG777516	*Cladonia uncialis*	KS-AT-DH-MT-ER-KR-ACP	MT, SDR, p450, FAD	Genome sequencing	–	–	[15, 16]
64	*Eppks6*	ERF74612	*Endocarpon pusillum*	KS-AT-DH-MT-ER-KR-ACP	–	Genome sequencing	–	–	[110]
65	*Eppks7*	ERF70234	*Endocarpon pusillum*	KS-AT-DH-MT-ER-KR-ACP	–	Genome sequencing	–	–	[110]
66	*Eppks8*	ERF71956	*Endocarpon pusillum*	KS-AT-DH-MT-ER-KR-ACP	–	Genome sequencing	–	–	[110]
67	*Eppks9*	ERF77204	*Endocarpon pusillum*	KS-AT-DH-MT-ER-KR-ACP	–	Genome sequencing	–	–	[110]
68	*Pmpks8*	HM180411	*Peltigera membranacea*	KS-AT-DH-MT-ER-KR-ACP	–	Genome sequencing	–	–	[61]
69	*Pmpks9*	HM180412	*Peltigera membranacea*	KS-AT-DH-MT-ER-KR-ACP	–	Genome sequencing	–	–	[61]
70	*Scpks1*	EF554834	*Solorina crocea*	KS-AT-DH-MT-ER-KR-ACP	–	gDNA—library screening	–	–	[42]
71	*Cu-r-pks-15*	MG777513	*Cladonia uncialis*	KS-AT-DH-MT-KR-ACP-R	SDR, FAD, p450, De[H]	Genome sequencing	Betaenone A-C	Homology mapping	[15, 16]
72	*Pmpks1*	GU441232	*Peltigera membranacea*	KS-AT-DH-ER-KR	–	Genome sequencing	–	–	[61]
73	*Pmpks2*	GU460164	*Peltigera membranacea*	KS-AT-DH-ER-KR	–	Genome sequencing	–	–	[61]
74	*Pmpks3*	GU477350	*Peltigera membranacea*	KS-AT-DH-ER-KR	–	Genome sequencing	–	–	[61]
75	*Pmpks10*	HM189674	*Peltigera membranacea*	KS-AT-DH-ER-KR	–	Genome sequencing	–	–	[61]
76	*Eppks10*	ERF73749	*Endocarpon pusillum*	KS-AT-DH-MT-ER-KR	–	Genome sequencing	–	–	[110]
77	*Pmpks5*	HM180408	*Peltigera membranacea*	KS-AT-DH-MT-ER-KR	–	Genome sequencing	–	–	[61]
78	*Pmpks7*	HM180410	*Peltigera membranacea*	KS-AT-DH-MT-ER-KR	–	Genome sequencing	–	–	[61]
79	*Pmpks11*	HM189675	*Peltigera membranacea*	KS-AT-DH-MT-KR-ACP-C	–	Genome sequencing	–	–	[61]

PKS gene abbreviations: Ketosynthase (KS)|Acyltransferase (AT)|Dehydratase (DH)|Ketoreductase (KR)| C-methyltransferase (MT)|Enoylreductase (ER)|Acyl carrier protein (ACP)|Reductase (R), Condensation domain (C) Accessory gene abbreviations: No information available (–)|No accessory genes identified ([none])|L-amino acid oxidase (LAAO)|Monooxygenase (Mono)|Unknown func-

Table 2 (continued)

tion (Unk)|Cytochrome p450 oxidase (p450)|Short-chain dehydrogenase/reductase (SDR)|O-methyltransferase (MT)|Oxidoreductase (OR)|Thioesterase (TE)|Reductase (Red)|Dehydrogenase (De[H])|Decarboxylase (De[CO2])|Hydratase (Hyd)|Alanine ligase (AlaLig)|Dehydratase (De[H2O])|Carboxylesterase (CE)|Glucose-methanol-choline oxidoreductase (GMC)|Oxidase ([O])|Aldehyde dehydrogenase (AldDe[H])

Table 3 Type III polyketide synthase genes in lichenizing fungi

Entry	Name	Accession	Species	Putative tailoring enzymes	Detection method	Putative function	Prediction method	Ref(s)
80	*Cu-t3-pks-1*	MG777469	*Cladonia uncialis*	[None]	Genome sequencing	Chalcone synthase	BLAST consensus	[15, 16]
81	*Cu-t3-pks-2*	MG777470	*Cladonia uncialis*	p450	Genome sequencing	Chalcone synthase	BLAST consensus	[15, 16]

Accessory gene abbreviations: no accessory genes identified ([None])|Cytochrome p450 oxidase (p450)

The majority of non-reducing PKS (26 out of 38) possess terminal thioesterase (TE) domains (Table 1). The TE domain in fungi liberates polyketides by thioester hydrolysis and is the most common termination method by PKS [37]. Four non-reducing PKS possess Claisen cyclase (CYC) domains (Entries 27–30). This is an alternative mode of termination used by a minority of PKS. In these PKS, a Claisen-style mechanism is used to simultaneously cyclize and release the polyketide [40]. Aflatoxin and sterigmatocystin are examples of fungal polyketides requiring CYC-mediated termination [71, 120]. Methylphloroacetophenone is a lichen polyketide hypothesized to be the product of CYC-mediated release by CU-NR-PKS-2 (Entry 27) in *C. uncialis* [3]. Five non-reducing PKS and one reducing PKS possessed a terminal reductase (R) domain (Entries 31–35 in Table 1; Entry 71 in Table 2). This terminal domain releases polyketides via NADPH-dependent reduction of thioesters resulting in aldehydes. This mechanism was first observed in *Acremonium strictum* [12]. We, therefore, hypothesize that lichen PKS-possessing reductase domains produce polyketides containing aldehydes. It is unclear whether these aldehydes are chemically altered by tailoring enzymes encoded near the PKS.

GCN5-related *N*-acetyltransferases (GNAT) may prime ACP domains with acetyl-CoA instead of an SAT domain or loading module. These acetyltransferases may also function as a post-synthetic tailoring enzyme by acylating nascent polyketides [48, 107]. A single GNAT was observed encoded near *Cu-nr-pks-12* (Entry 28) of *C. uncialis* [15]. The presence of a SAT domain on *Cu-nr-pks-12* suggests that the function of this GNAT involves acylation of a polyketide rather than priming polyketide assembly. Functional characterization of this putative GNAT would demonstrate the participation of this class of enzyme in lichen secondary metabolite biosynthesis.

Three non-reducing PKS, encoded as *Cu-nr-pks-13*, *Ulpks5*, and *Xsepks1*, did not possess terminal domains (Entries 36–38). We were unable to observe a terminal domain with BLAST [8] and AntiSMASH (v. 4.0) [20]. Some fungal non-reducing PKS, such as the PKS responsible for pestheic acid biosynthesis in *Pestalotiopsis fici*, do not have terminal release domains [117]. In this case, a β-lactamase is proposed to replace the role of a terminal release domain by facilitating thioester hydrolysis and Claisen cyclization [117]. A biosynthetic role for *Cu-nr-pks-13* (Entry 36) has been previously proposed to produce an intermediate of the pestheic acid biosynthetic pathway [16]. This proposal is based on the observation of genetic similarity and common domain architecture of these PKS as well as the presence of a β-lactamase encoded near *Cu-nr-pks-13* (vide infra) [16]. It is noteworthy that a β-lactamase was found near *Ulpk5* in *Usnea longissima* (Entry 37) and that phylogenetic analysis suggested that *Ulpks5* is associated with anthraquinone biosynthesis [115]. These observations would be consistent with a biosynthetic function for ULPKS5 involving anthraquinone intermediates of the pestheic acid biosynthetic pathway.

A total of 41 reducing PKS genes from lichenizing fungi are catalogued in Table 2. Eight of these PKS are partially reducing PKS because they lack enoylreductase (ER) domains (Entries 39–44, 71, 79). An example of a metabolite produced by a PKS lacking ER in non-lichenizing fungi is 6-methylsalicylic acid, the first intermediate in patulin biosynthesis [85]. PKS genes encoding 6-methylsalicylic acid synthases appear to be widely distributed among lichenizing fungi [88]. A 6-methylsalicylic acid synthase associated with what appears to be a complete patulin gene cluster was observed in *C. uncialis* (vide infra) [16].

Seven reducing PKS listed in Table 2 do not possess an acyl carrier protein (ACP) domain (Entries 72–78). This is an unusual observation because, to our knowledge, there are no examples of PKS lacking ACP nor of *trans*-ACP proteins servicing type I iterative PKS. These ACP-less PKS were observed in genome sequencing of *Peltigera membranacea*

and *Endocarpon pusillum* [61, 110]. It is possible that these PKS were inactivated by evolution and have been rendered non-functional though loss of ACP domains. As complete annotation of PKS genes was unnecessary to meet the research objectives of both studies, it is also possible that ACP domains exist but were inadvertently omitted from the GenBank records [61, 110].

One PKS annotated in *P. membranacea* (Entry 79) does possess an ACP but also possesses a condensation (C) domain similar to those of non-ribosomal peptide synthases (NRPS). We again speculate that either this PKS was rendered non-functional by evolution or is a hybrid PKS–NRPS with an incomplete GenBank entry. This gene, ambiguously a PKS or a PKS–NRPS, is included in this review for completeness. A total of five PKS–NRPS genes have been annotated in lichenizing fungi, three from *C. uncialis* and two from *E. pusillum*. These hybrid PKS–NRPS were not included in this review but information on PKS–NRPS genes in lichens can be found elsewhere [15, 110].

Methods of finding PKS genes

The use of degenerate primers to amplify PKS gene fragments from genomic DNA templates is the most commonly applied method for discovering PKS genes in lichenizing fungi [69, 70, 75]. It has been shown that degenerate primers can be used to target specific PKS phylogenetic clades or functionalities [17, 75]. The subsequent sequence information can then be used to design probes to screen genomic DNA libraries through Southern blot. The PKS genes obtained using degenerate primers on genomic DNA templates are listed as 'gDNA—library screening' in Tables 1 and 2. An advantage of screening genomic DNA libraries is the possibility of gaining sequence information on both the PKS and surrounding accessory tailoring genes. Retrobiosynthetic deductions could then be applied on a known polyketide to ascertain whether the functions of the identified PKS and accessory enzymes are compatible with a plausible biosynthetic pathway. Information on accessory tailoring genes is, therefore, useful if the purpose of the study is to identify which gene cluster, from among a list of candidate clusters, is most likely to be responsible for the biosynthesis of a known metabolite (e.g., [3]).

Using cDNA-based templates generated from reverse-transcribed mRNA is another method of identifying new PKS genes. For example, degenerate primers were used to target PKS gene fragments in *Cladonia grayi* [11]. The DNA sequencing results were consolidated with a preliminary genome assembly to obtain complete PKS genes. These PKS genes are listed as 'cDNA—genome assembly' under entries 1, 7, 8, 9, 29, 30 of Table 1. Targeting transcriptionally active PKS genes through the rapid amplification of cDNA end (RACE) technique [119] can also be used to propose biosynthetic functions for the encoded PKS [2, 23]. In one case, application of RACE-PCR was followed by genome sequencing to obtain information on accessory tailoring genes (Table 1, Entry 13). These PKS genes are listed as 'cDNA—RACE' under entries 13, 23, and 24 in Table 1. One of the key advantages of cDNA-based methods is that transcriptionally active PKS are more likely to be responsible for the biosynthesis of identifiable polyketides within organic extracts of lichen tissue. Observing that a PKS gene is transcriptionally active does not guarantee an association with an identifiable metabolite because protein expression may be down-regulated by post-transcriptional regulatory processes. The slow metabolism of lichens may also introduce a time-lag between transcription of a PKS gene and the emergence of detectable quantities of the secondary metabolite.

The emergence of 'next-generation' sequencing technologies has recently enabled the rapid detection of PKS and accessory genes from raw read assemblies [24, 36, 44, 126]. An Illumina platform was used to construct draft assemblies of the mycobiont and photobiont genomes of the lichen *P. membranacea* [61]. The detection of putative PKS gene clusters was achieved using BLAST [8] consensus alignments of KS and ACP domains. This approach yielded 11 PKS genes within the mycobiont [61]. The genome of *E. pusillum* was sequenced using both Roche 454 and Illumina platforms, yielding 15 PKS genes [110]. An Illumina platform was recently used to sequence the sub-cultured mycobiont of *C. uncialis*, yielding 32 type I PKS and 2 type III PKS [15]. PKS genes identified by genome sequencing are listed as 'genome sequencing' in Tables 1, 2, 3. With lower costs and higher throughput, it is likely that whole-genome sequencing will be the method of choice for future PKS gene surveying studies in lichens.

Methods of predicting PKS functions

Phylogenetics is a useful tool to predict the biosynthetic function of gene products [127]. A core premise of phylogenetics is that genes with common ancestry that evolved within a recent timeframe are more likely to encode proteins with similar functions compared to related genes within a distant timeframe. In secondary metabolite biosynthetic studies, observation of common and recent ancestry between a subject and reference gene is used as supporting evidence that the encoded proteins possess similar roles in metabolite biosynthesis. In the example of modular *trans*-AT PKS, phylogenetic analyses can predict polyketide structure more accurately than reviewing PKS domain architecture [74]. Phylogenetics can be, therefore, used to provide proposals of function for PKS genes in lichenizing fungi [88, 103, 105]. For example, three PKS in *U. longissima*, encoded as *ulpks2*, *ulpks4*, and *ulpks6* (Entries 6, 22, 26), had putative

functions assigned using this approach [113]. Phylogenetic analyses revealed that these three genes were related to *mpac*, a 6-methylorsellinic acid synthase from *Penicillium brevicompactum*, *mos*, a 3-methylorcinaldehyde synthase from *Acremonium strictum*, and *an7909*, an orsellinic acid synthase from *Aspergillus nidulans*. It was, therefore, proposed that these PKS were responsible for the biosynthesis of 5-methylorsellinic acid, 3-methylorcinaldehyde, and orsellinic acid, respectively [113]. The PKS genes for which phylogenetics were used to propose putative functions are listed in Tables 1 and 2 as 'Phylogenetics'.

Transcription profiling was used to assign *Cgrpks16* as the PKS gene responsible for grayanic acid biosynthesis in *C. grayi* [11]. First, incubation conditions were established to reproducibly induce grayanic acid production in *C. grayi* [33]. Quantitative PCR of candidate PKS genes demonstrated a correlation between increased levels of *Cgrpks16* transcription and grayanic acid bioaccumulation over a period of 36 days. A phylogenetic analysis demonstrated genetic similarity between *Cgrpks16* and a PKS from a non-lichenizing fungus known to produce orsellinic acid-based metabolites. As grayanic acid biosynthesis requires orsellinic acid as the first intermediate, this phylogenetic analysis supports the assignment of CgrPKS16 as the PKS responsible for grayanic acid biosynthesis. Genes encoding a cytochrome p450 and an *O*-methyltransferase were found to be located near *Cgrpks16*, enabling the authors to propose a complete grayanic acid biosynthetic pathway [11]. This 'Transcription profiling' approach is listed under entry 9 of Table 1.

A gene surveying approach was used in the assignment of *Cu-nr-pks-2* as the encoded PKS responsible for usnic acid biosynthesis in *C. uncialis* [3]. Radiolabelling and oxidation experiments [13, 101] suggested that usnic acid is formed from two molecules of methylphloroacetophenone. This indicates that the usnic acid-associated PKS requires a *C*-methyltransferase (MT) and Claisen cyclase (CLC) domains. An oxidative enzyme such as a cytochrome p450 oxidase would be required to dimerize methylphloroacetophenone into usnic acid. By sequencing the mycobiont genome and running a preliminary assembly through AntISMASH (v. 2.0) [19], a total of 32 candidate PKS were identified [3]. Only one candidate PKS, *Cu-nr-pks-2*, was consistent with these biosynthetic requirements and was, therefore, named methylphloroacetophenone synthase (*mpas*) [3]. Reverse transcriptase-PCR demonstrated that *mpas* was transcriptionally active in a strain of *C. uncialis* where usnic acid was the only observable secondary metabolite [3]. This 'gene surveying' approach is listed under entry 27 in Table 1.

A homology mapping approach was adopted to propose that the PKS encoded as *Cu-nr-pks-8* (Table 1, Entry 13) in *C. uncialis* produces a halogenated isocoumarin [2]. Three genes within the cluster of *Cu-nr-pks-8* were observed to be genetically similar to three genes within the terrein biosynthetic gene cluster in *Aspergillus terreus* [123]. In *A. terreus*, these genes encode a PKS that is responsible for producing 2,3-dehydro-6-hydroxymellein, a *trans*-KR-DH-like protein that reduces this first intermediate to form 6-hydroxymellein, and a monooxygenase responsible for the oxidation of 6-hydroxymellein to an unidentified intermediate. It was, therefore, proposed that the biosynthetic functions of the lichen PKS, the *trans*-KR-DH-like protein, and the monooxygenase in *C. uncialis* are identical to those of *A. terreus* and are, therefore, responsible for producing an isocoumarin [2]. In *C. uncialis*, this isocoumarin is not derivatized to produce terrein, but instead appears to be halogenated and *O*-methylated into an unknown final product. These modifications were proposed because genes encoding a halogenase and an *O*-methyltransferase were found near the PKS (Entry 13, Table 1) [2]. This homology mapping approach was also applied to propose that three PKS gene clusters in *C. uncialis* are responsible for the biosynthesis of grayanic acid (Table 1, Entries 12), patulin (Table 2, Entry 41), and betaenone A–C (Table 2, Entry 71) [16]. Pathway intermediates, but not final metabolites, were proposed for four additional *C. uncialis* PKS gene clusters. In these latter cases, similar to the example of *Cu-nr-pks-8* and terrein biosynthesis, it has been proposed that *C. uncialis* and the reference organisms produce polyketide intermediates that are common to both organisms. These common intermediates would then be derivatized by tailoring enzymes that do not appear to share similar functions to both organisms, resulting in the production of final products that are distinct to both species. In *C. uncialis*, these final products are unknown but are proposed to be related to the pathway intermediates of azaphilone (Table 1, Entry 15), fusarubin Table 1, Entry 25), pestheic acid (Table 1, Entry 34), and mycophenolic acid (Table 1, Entry 36) [16]. The PKS genes with putative functions identified through this 'homology mapping' approach are found in Tables 1 and 2.

Prospects for genomics-based natural product discovery

Genome sequencing is an emerging method to find lichen biosynthetic genes. Genome sequencing projects among bacteria, plants, and fungi have revealed that many more biosynthetic gene clusters are encoded within organisms than the number of secondary metabolites known to be produced by them [5, 49, 76, 89]. A similar case is apparent for lichens [15, 61, 110]. How can the function of these cryptic genes be determined? The sensitivity of an organism's chemical profile to a new stimulus can be exploited to activated otherwise silent genes [21]. The effect of changing environmental conditions on lichens (e.g., carbon source, desiccation,

UV radiation) is explored for this reason [22, 35, 38, 98, 99, 104]. Inducing grayanic acid production to identify its gene cluster in *C. grayi* is an example of the utility of these experiments [33]. Among non-lichenized life, new natural products have been discovered using ribosome engineering [54], mixed organism culturing [77], induction by rare-earth elements [102], and overproducing malonyl-CoA [118, 122]. Manipulation of transcriptional regulators [4, 100] and the recent application of CRISPR–Cas9 to activate a silent gene cluster [125] demonstrate the utility of genetic engineering towards natural products discovery. To apply techniques such as promoter modification, it would first be necessary to develop methods of modifying the lichen genome. At present, only one method of DNA modification has been reported: a relatively fast-growing species of lichen named *Umbilicaria muehlenbergii* was modified by random insertional mutagenesis using a tumour-inducing plasmid produced by the plant pathogen *Agrobacterium tumefaciens* [78]. This technique would allow researchers to screen a library of lichens subjected to random gene knockouts and identify interesting emergent phenotypes for further study. Nonetheless, we are still a long way away from developing a genetic toolbox for lichens that is comparable to the vast repertoire of techniques and procedures currently available for model bacteria and fungi. Co-cultivation of lichens with other organisms or cultivation in the presence of rare-earth metals are examples of techniques that do not require such a genetic toolbox. We anticipate that these latter techniques will be used more often in the foreseeable future.

It is notable that most compiled PKS gene clusters do not have polyketides associated with them. Functional heterologous expression of lichen PKS genes in host organisms will likely be required to identify the associated polyketides. There are no examples of heterologous expression experiments yielding metabolites from lichen PKS genes. However, transcription of transformed PKS genes including intron removal has been reported on three occasions. The first example was the transformation of *A. nidulans* with the PKS gene *xsepks1* from *Xanthoparmelia semiviridis* [29]. Using the native *X. semiviridis* promoter, the transcription of *xsepks1* including the excision of a 63-bp intron was observed. Similar results were reported when *Aspergillus oryzae* was transformed with the PKS gene *scpks1* from *Solorina crocea*, then transcribed using a host promoter [42]. More recently, the *U. longissima* PKS gene *ulpks6* was transformed into *A. nidulans* [114] and was observed to be transcriptionally active when using a host promoter. Nonetheless, de novo polyketide biosynthesis was not observed in any case.

The challenges associated with the production of lichen polyketides in *Aspergillus* hosts have motivated a recent investigation into possible systematic causes. Though the cause of this problem remains unknown, the ketosynthase domains of lichen PKS appear similar in structure and function to those of non-lichenizing fungi and was, therefore, ruled out as a potential source of this problem [1]. PyrG is a decarboxylase found in the lichen *S. crocea* and is responsible for the decarboxylation of orotidine 5′-monophosphate to the primary metabolite uridine monophosphate. Functional heterologous expression of PyrG including product turnover was achieved in *A. nidulans* [94]. This experiment provides proof-of-concept evidence that heterologous expression of lichen biosynthetic genes is possible.

The lichen genome is an unexplored frontier and it is anticipated that genome sequencing will greatly facilitate the discovery and assignment of biosynthetic gene clusters. The key technological breakthrough that will accelerate natural product discovery in lichenizing fungi will be the development of a stable platform for the heterologous expression of lichen biosynthetic gene clusters in a non-lichen host.

Author's note

During manuscript revision, a transcriptomic study of PKS genes in *Nephromopsis pallescens* was reported, revealing 16 PKS genes in this lichen. The authors used phylogenetic and qPCR analyses to identify a putative usnic acid PKS among candidate genes [111]. The experimental approach has parallels with the 'transcription profiling' approach used to identify the grayanic acid gene cluster in *C. grayi* (described in text). The draft genome of *Lasallia hispanica* was also recently reported, revealing 19 PKS genes in this lichen [34]. However, these articles appeared in the literature during final revisions on this manuscript. The authors elected to not re-write the manuscript to include these recent publications.

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Compliance with ethical standards

Conflict of interest There are no conflicts of interests to declare.

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