ELSEVIER

British Mycological Society promoting fungal science

journal homepage: www.elsevier.com/locate/mycres

6-MSAS-like polyketide synthase genes occur in lichenized ascomycetes

Imke SCHMITT[a,b,*], *Stefanie KAUTZ*[c], *H. Thorsten LUMBSCH*[a]

[a]*Department of Botany, The Field Museum, 1400 South Lake Shore Drive, Chicago, IL 60605, USA*
[b]*Leibniz-Institut für Naturstoff-Forschung und Infektionsbiologie, Hans-Knöll-Institut, Beutenbergstraße 11a, D-07745 Jena, Germany*
[c]*Fachbereich Biologie und Geografie, Universität Duisburg-Essen, Campus Essen, Universitätsstraße 5, D-45517 Essen, Germany*

ARTICLE INFO

Article history:
Received 20 December 2006
Received in revised form
3 July 2007
Accepted 29 August 2007
Corresponding Editor: Marc Stadler

Keywords:
Ascomycota
Evolution
Lichens
Phylogeny
Polyketide biosynthesis
Secondary metabolites

ABSTRACT

Lichenized and non-lichenized filamentous ascomycetes produce a great variety of polyketide secondary metabolites. Some polyketide synthase (PKS) genes from non-lichenized fungi have been characterized, but the function of PKS genes from lichenized species remains unknown. Phylogenetic analysis of keto synthase (KS) domains allows prediction of the presence or absence of particular domains in the PKS gene. In the current study we screened genomic DNA from lichenized fungi for the presence of non-reducing and 6-methylsalicylic acid synthase (6-MSAS)-type PKS genes. We developed new degenerate primers in the acyl transferase (AT) region to amplify a PKS fragment spanning most of the KS region, the entire linker between KS and AT, and half of the AT region. Phylogenetic analysis shows that lichenized taxa possess PKS genes of the 6-MSAS-type. The extended alignment confirms overall phylogenetic relationships between fungal non-reducing, 6-MSAS-type and bacterial type I PKS genes.

Introduction

Lichenized fungi produce a great variety of secondary metabolites. These compounds could be useful as pharmaceuticals (e.g. Miao *et al.* 2001) or as pesticides (Dayan & Romagni 2001), and thus hold an unexploited remedial and economical potential. Most lichen substances are small aromatic polyketides synthesized by the fungal partner in the symbiosis. Commonly encountered lichen products are esters or products of oxidative coupling derived from the simple aromatic ring compounds orcinol or β-orcinol. These include orcinol depsides, β-orcinol depsides, orcinol depsidones, β-orcinol depsidones, and depsones (e.g. Fig 1: compounds 3–8). These compounds occur almost exclusively in lichenized fungi. They are predominantly deposited on the medullary hyphae in the lichen thallus, and may play a role in the regulation of water content and carbon dioxide diffusion due to their hydrophobic properties (Huneck 2002; Lange *et al.* 1997; Souza-Egipsy *et al.* 2000). Other substance classes derived by the polyketide pathway, such as xanthones (Fig 1: compounds 1,2), anthraquinones (Fig 2: compound 9), chromones, and fatty acids are not peculiar to lichens. Norsolorinic acid (Fig 2: compound 9), for example, is a precursor in sterigmatocystin and aflatoxin biosynthesis found in several *Aspergillus* species (e.g. Brown *et al.* 1996).

Secondary metabolites play an important role in the taxonomy of lichen-forming ascomycetes and are employed at different taxonomic levels from species and subspecific to

* Corresponding author. University of Minnesota, Department of Plant Biology, 250 Biological Sciences Center, 1445 Gortner Ave., St. Paul, MN 55108, USA.
E-mail address: schm2109@umn.edu
0953-7562/$ – see front matter © 2007 The British Mycological Society. Published by Elsevier Ltd. All rights reserved.
doi:10.1016/j.mycres.2007.08.023

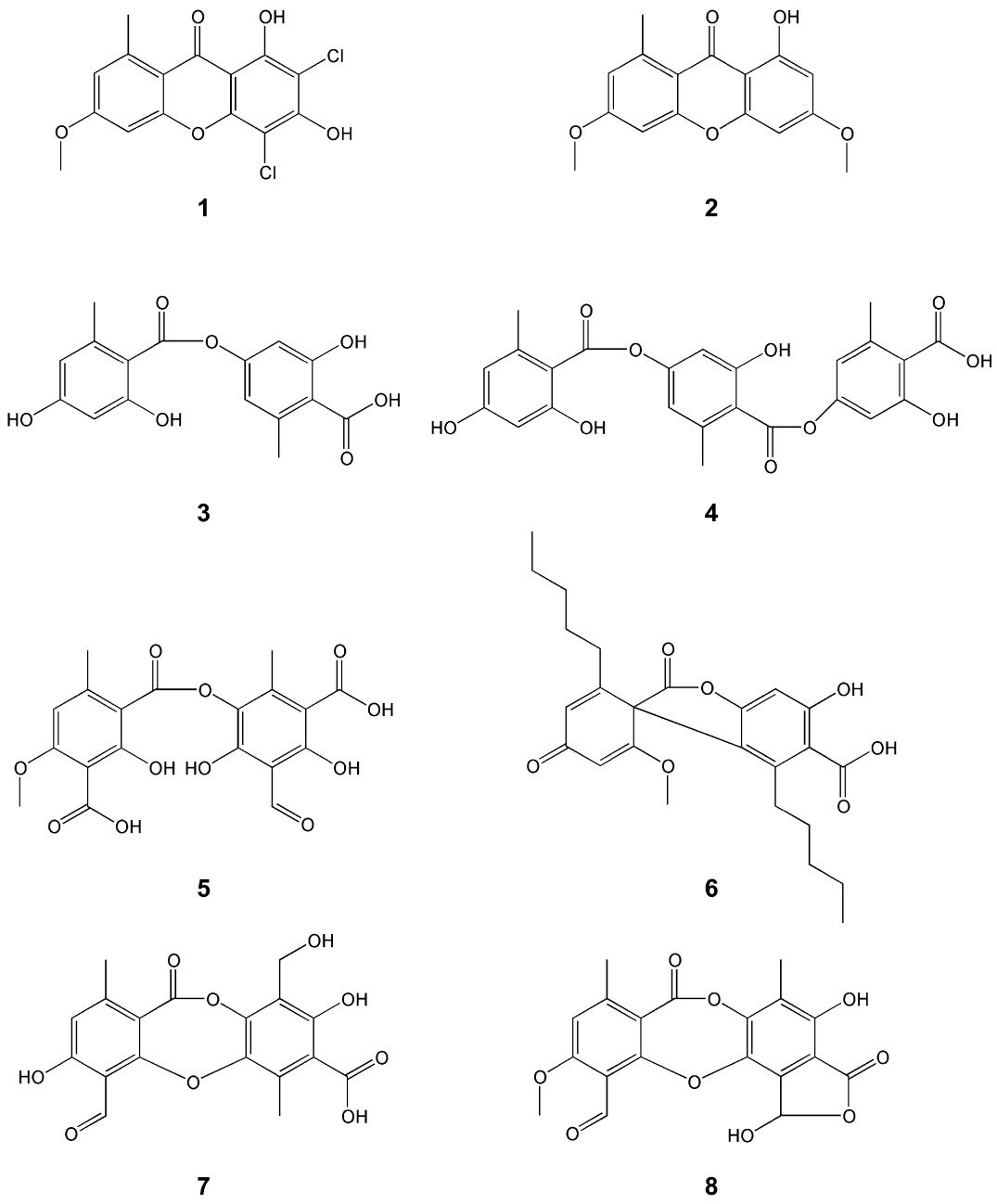

Fig 1 – Secondary metabolites from lichenized fungi: xanthones: thiophaninic acid (1), lichexanthone (2); orcinol depsides: lecanoric acid (3), gyrophoric acid (4), thamnolic acid (5); depsones: picrolichenic acid (6); β-orcinol depsidones: protocetraric acid (7), stictic acid (8).

generic and higher-ranks (Hawksworth 1976; Lumbsch 1998a, b). In this connection the presence or absence of single substances or groups of biosynthetically related compounds, so-called chemosyndromes, is analysed (Culberson & Culberson 1977). Traditionally, hypotheses about biogenetical relationships between lichen compounds have been deduced from comparison of the chemistries of closely related taxa (Culberson 1964; Culberson & Culberson 1970), and from theoretical considerations based on the chemical synthesis of the metabolites (Elix *et al.* 1987). Today, it is possible to analyse phylogenetic relationships of the involved biosynthetic genes directly.

Polyketide biosynthesis in fungi is achieved by large multifunctional proteins, so-called polyketide synthases (PKSs). These enzymes assemble structurally diverse products from simple acyl-CoA substrates by using a catalytic cycle involving decarboxylative Claisen condensations and variable modifications, such as reduction and dehydration. Fungal aromatic PKSs have iterative reaction cycles, where catalytic domains are used repetitively in a specific order programmed by the protein. Fungal PKS genes possess keto synthase (KS), acyl transferase (AT), and acyl carrier protein (ACP) domains. Additionally they may have one or two more ACPs, an integrated methyl transferase (ME), a Claisen

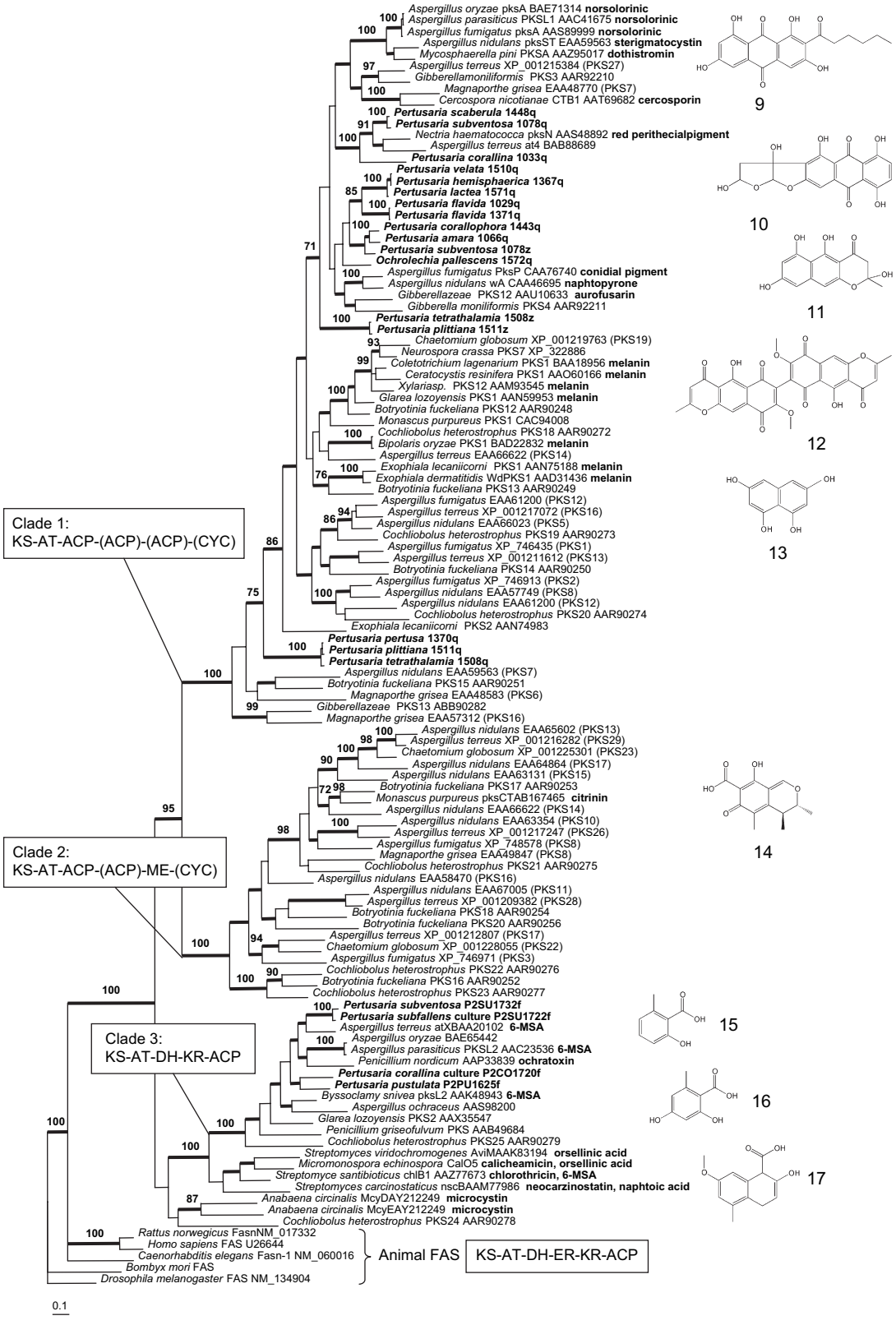

Fig 2 – Phylogeny of fungal and bacterial type I PKS genes based on partial keto synthase (KS), complete linker, and partial acyl transferase (AT) region. This is a 50 % majority rule consensus tree based on 36 K trees from a Bayesian analysis. Bold branches indicate posterior probabilities equal or above 0.95. Numbers above branches are maximum parsimony BS support values greater than 70 % based on 2 K pseudoreplicates. New sequences from lichenized fungi are indicated in bold print. Genbank accession numbers, PKS gene designation, and function (if known) are given. Structures to the right represent example gene products: norsolorinic acid (9), dothistromin (10), naphtopyrone (11), aurofusarin (12), 1,3,6,8-tetrahydroxy-naphthalene (13), citrinin (14), 6-methylsalicylic acid (6-MSA) (15), orsellinic acid (16), and naphtoic acid (17).

cyclase (CYC), and reducing domains, such as keto reductase (KR), dehydratase (DH), and enoyl reductase (ER). Phylogenetic studies based on alignments of the KS region show that fungal PKSs group into reducing, non-reducing, and partially reducing (6-MSAS-type) PKSs (Kroken *et al.* 2003; Nicholson *et al.* 2001).

Ascomycete genomes possess many PKS genes (Table 2), but very few have been functionally characterized. Thus, phylogenetic analysis of KS domains constitutes a tool to sort PKS genes, make predictions about the presence or absence of particular active sites downstream of the KS, and narrow down the number of potentially synthesized compound groups. As typical lichen substances are non-reduced aromatic polyketides consisting of simple or multimembered aromatic rings, they are most likely produced by non-reducing PKS genes. Indeed, several PKS paralogues of putatively non-reducing PKSs are found in lichenized fungi, although there is no obvious correlation between secondary metabolite and PKS clade (Grube & Blaha 2003; Schmitt *et al.* 2005). A further group of PKS genes, which is interesting in connection with lichen compounds, is a group of fungal PKSs involved in the production of 6-methylsalicylic acid (6-MSA). Biosynthesis of 6-MSA is catalysed by iterative type I PKSs (KS-AT-DH-KR-ACP) in fungi (Beck *et al.* 1990) and in bacteria (Jia *et al.* 2006). 6-MSA is assembled from one acetyl-coenzyme A (CoA) and three malonyl CoAs to form a linear tetraketide. The KR domain offers a selective ketoreduction at the C-5 position. The resultant unsaturated intermediate undergoes an intramolecular aldol condensation to furnish the 6-MSA structure (reviewed by Staunton & Weissmann 2001). Orsellinic acid in bacteria is synthesized by a highly similar PKS, which lacks the KR domain and thus produces the saturated orsellinic acid structure (Ahlert *et al.* 2002; Weitnauer *et al.* 2001). In fungi orsellinic acid synthases have not yet been identified. Previous phylogenetic analyses of type I PKS in bacteria and fungi suggest a close relationship between bacterial orsellinic acid PKSs and fungal 6-MSA PKSs (Kroken *et al.* 2003). Thus it is conceivable that orcinol or orsellinic acid as typical base units of lichen depsides, depsidones, and depsones could be synthesized by a PKS similar to fungal 6-MSAS.

The aim of the present study is to detect PKS genes in mycobiont genomes that are potentially involved in the production of typical lichen substances. We use a degenerate primer approach to detect non-reducing PKS, and fungal PKS involved in the biosynthesis of small aromatic compounds of the 6-MSA-type. We selected lichenized fungi of the *Pertusariales* as a model group, as members of this order are known to produce various secondary metabolites, such as chlorinated and non-chlorinated xanthones, orcinol depsides, orcinol depsidones, β-orcinol depsidones, and depsones (e.g. Archer 1993; Brodo 1991; Dibben 1980; Hanko 1983; Hanko *et al.* 1985) (Table 1). Also, the molecular phylogenetic relationships of members of this order are well established (Lumbsch *et al.* 2006; Schmitt & Lumbsch 2004; Schmitt *et al.* 2006). The KS domain is the most conserved region of the PKS and is typically used for phylogenetic analyses (e.g. Grube & Blaha 2003; Kroken *et al.* 2003; Sauer *et al.* 2002; Schmitt *et al.* 2005). In the current study we explore the phylogenetic utility of a longer PKS fragment spanning most of the KS domain, the

Table 1 – Lichenized fungi and mycobiont cultures used in the current study

Organism	Source	No. of PKS found	GenBank accession and clone no.	Major secondary compound in sample
Ochrolechia pallescens	Spain, *Lumbsch* 2004 (F)	1	EF192111 (1572q)	Gyr, var
Pertusaria amara	Canada, 20 Aug 2003, *Lumbsch, Schmitt, Wirtz* (F)	1	EF192116 (1066q)	Pic, protocet
P. corallina	Spain, 5 Jun 2003, *Schmitt* (F)	1	EF192117 (1033q)	Tham
P. corallina	Culture 1118 M (AKITA)	1	EF192112 (1720f)	Tham
P. corallophora	Antarctica, *Lumbsch* 19013 d (F)	1	EF192118 (1443q)	Protocet
P. flavida	Spain, 5 Jun 2003, *Schmitt* (F)	1	EF192119 (1029q)	Thio, st
	Germany, 15 Apr 2004, *Schmitt* (F)	1	EF192120 (1371q)	Thio, st
P. hemisphaerica	Germany, 15 Apr 2004, *Schmitt* (F)	1	EF192121 (1367q)	Lec
P. lactea	Spain, *Lumbsch*, 2004 (F)	1	EF192122 (1571q)	Lec, var
P. pertusa	Germany, 15 Apr 2004, *Schmitt* (F)	1	EF192123 (1370q)	4,5-Dichl, st
P. plittiana	USA, Michigan, 6 Jun 2004, *Lumbsch & Kautz* (F)	2	EF192124 (1511q)	Norst, perlat, steno
			EF192125 (1511z)	
P. pustulata	Japan, *Yamamoto* 15030102 (AKITA)	1	EF192113 (1625f)	St, 2-chl
P. scaberula	Australia, *Archer* P932 (NSW)	1	EF192126 (1448q)	Lich, tham
P. subfallens	Culture 1086M (AKITA)	1	EF192114 (1722f)	Pic
P. subventosa	Australia, *Lumbsch* 19070a (F)	2	EF192127 (1078q)	Lich, pic, tham
			EF192128 (1078z)	
	Peru, *Lumbsch, Ramirez, Wirtz* 19351f (F)	1	EF192115 (1732f)	Lich, pic, tham
P. tetrathalamia	USA, Michigan, *Lumbsch* 19191b and *Kautz* (F)	2	EF192129 (1508q)	4,5-Dichl, st
			EF192130 (1508z)	
P. velata	USA, Michigan, *Lumbsch* 19198a and *Kautz* (F)	1	EF192131 (1510q)	Lec, lich

Abbreviations for secondary substances: gyr, gyrophoric acid; lec, lecanoric acid; lich, lichexanthone; norst, norstictic acid; perlat, perlatolic acid; pic, picrolichenic acid; protocet, protocetraric acid; st, stictic acid; steno, stenosporic acid; tham, thamnolic acid; thio, thiophaninic acid; var, variolaric acid; 2-chl, 2-chloro-6-O-methylnorlichexanthone; and 4,5-dichl, 4,5-dichlorolichexanthone.

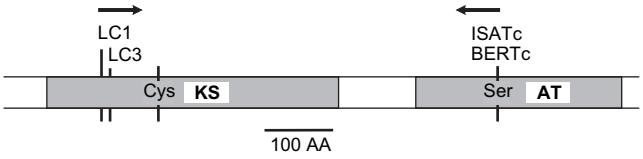

Fig 3 – Position of degenerate PKS primers used in this study. Locations of active site cystein (Cys) residue in the keto synthase (KS) domain, and serine (Ser) in the acyl transferase (AT) are given.

linker region between KS and AT, and part of the AT domain (Fig 3).

Material and methods

Taxon sampling

Lichen and mycobiont material used in the current study is summarized in Table 1. Mycobiont cultures were obtained from Yoshikazu Yamamoto (Akita Prefectural University, Japan) and cultivated on MY medium (Yamamoto *et al.* 2002) at room temperature. To produce a comprehensive alignment of the new sequences and other fungal PKS genes we included PKS paralogues from nine ascomycete genomes. PKS sequences from the genomes of *Botryotinia fuckeliana, Cochliobolus heterostrophus, Gibberella moniliformis,* and *Neurospora crassa* were collected from a previous study (Kroken *et al.* 2003). In addition, we extracted PKS genes from the genomes of *Aspergillus fumigatus, A. nidulans, A. terreus, Chaetomium globosum,* and *Magnaporthe grisea*, using the sequence of *Botryotinia fuckeliana* PKS2 (GenBank accession AAR90238) as a BLAST template to screen individual genomes in Fungal Genomes Central (www.ncbi.nlm.nih.gov/projects/genome/guide/fungi/). We summarized the total number of detected PKS genes in Table 2. In the current study we used only those sequences that were fully alignable to the KS and AT domains, and that belong to the PKS groups of interest (fungal non-reducing and 6-MSAS-type PKS) as determined by preliminary phylogenetic analyses. All newly generated PKS genes from lichenized fungi, and random members of each of the detected clades (clades 1, 2, and 3) were then subjected to NCBI BLAST searches until most currently available PKS sequences, and especially all characterized genes, belonging to the clades of interest were found. We included four bacterial sequences showing strong homology to fungal 6-MSAS-type PKS, and two bacterial sequences showing some homology to PKS24 of *Cochliobolus heterostrophus* (AAR90278). Animal FAS were used as outgroups. A total of 101 fungal sequences were analysed, including 21 PKS genes from lichenized fungi.

Table 2 – Fungal genomes screened for polyketide synthase (PKS) genes

Species	PKS genes per genome	Sequences used in present study	
		Non-reducing PKS	6-MSAS-type
Aspergillus fumigatus	15	6	–
A. nidulans	24	12	–
A. terreus	29	9	1
Botryotinia fuckeliana	20	8	–
Chaetomium globosum	21	3	–
Cochliobolus heterostrophus	25	7	1
Gibberella moniliformis	15	2	–
Magnaporthe grisea	17	4	–
Neurospora crassa	7	1	–

Primers

We used degenerate primers of the LC series as forward primers in the KS region (Bingle *et al.* 1999), and developed new reverse primers in the AT domain. This primer combination amplified an approximately 1600–1700 bp fragment spanning most of the KS domain, the linker region, and half the AT domain (Fig 3). Primer design was based on the conserved amino acid motives HSLGEYA (non-reducing) and GHSVGEI (6-MSAS) around the active site serine residue of the AT domain. Sequences of the new degenerate primers are ISATc 5′-GCA TAI TCI CCI AGR CTR TG -3′, and BERTc 5′-ATY TCI CCI ACI GAR TGI CC-3′. Fungal non-reducing PKS genes can be amplified using the primer combinations LC1/ISATc, while 6-MSAS-type PKS genes can be amplified using LC3/BERTc.

DNA extraction, PCR, and sequencing

Total genomic DNA was extracted from finely ground lichen material or mycobiont thalli using the QIAGEN Plant Mini Kit (Qiagen, Valencia, CA). PCR reactions (25 μl) contained 2.5 μl dNTP mix, 1.25 μl of each primer (20 μM), 4 μl BSA, 0.25 μl AmpliTaq Gold (Applied Biosystems, Austin, TX), 2.5 μl Gold Buffer (Applied Biosystems), 2.5 μl $MgCl_2$ solution (25 mM), 5 μl genomic DNA extract and 5.75 μl water. Thermal cycling parameters were: initial denaturation for 10 min at 95 °C, followed by 33 cycles of 45 s at 95 °C, 45 s at 48 °C, 3 min at 72 °C, and a final elongation for 10 min at 72 °C. Bands of the expected size (1600–1700 bp) were excised from 1 % low melting point agarose TALE gels after electrophoresis. Agarose was digested using GELase (Epicentre Technologies, Madison, WI), and purified PCR products were ligated into the 2.1-TOPO cloning vector using TOPO TA cloning kits (Invitrogen, Carlsbad, CA). Multiple clones were sequenced from each ligation reaction using vector primers M13F and M13R, and the internal primers LC5 c or LC2 c (and forward versions thereof) (Bingle *et al.* 1999). Automated sequencing was carried out on an ABI 3730 DNA analyser (Applied Biosystems). Sequence fragments were assembled using SeqMan 4.03 (DNASTAR, Madison, WI) and manually adjusted. Vector sequences were trimmed off.

Sequence alignments and phylogenetic analysis

Parts of the AT domain and the linker region connecting the KS and AT domains are highly variable between PKS paralogues. Thus, amino acid sequences were aligned using a linear Hidden Markov Model (HMM) alignment procedure as

implemented in the software SAM (Sequence Alignment and Modelling) system (Karplus et al. 1998). This approach allows detection of remote sequence homologies and generation of reliable alignments even between dissimilar sequences. Regions that were not aligned with statistical confidence were excluded from phylogenetic analysis.

The alignment was analysed using maximum parsimony (MP) and a Bayesian approach (B/MCMC; Huelsenbeck et al. 2001; Larget & Simon 1999). MP analyses were performed using the program PAUP (Swofford 2003). A heuristic search using 100 random addition replicates was conducted with tree bisection–reconnection (TBR) branch swapping and MulTrees option in effect. Bootstrapping (Felsenstein 1985) was performed based on 2000 pseudoreplicates with the same settings as in the heuristic search.

The B/MCMC analyses were conducted using the MrBAYES 3.1.2. program (Huelsenbeck & Ronquist 2001). Posterior probabilities (PPs) were approximated by sampling trees using a MCMC method. The model of amino acid evolution was estimated among all fixed rate models implemented in MrBAYES by allowing model jumping. This procedure ensures that each model contributes to the results in proportion to its PP when the MCMC calculation has converged. Two parallel MrBAYES analyses were run for 2 M generations. In each analysis four chains were run simultaneously. Trees were sampled every 100th generation for a total of 20 K trees. The first 200 K generations (i.e. the first 2 K trees) were deleted as 'burn in' of the chain. We plotted the log-likelihood scores of sample points against generation time using TRACER 1.0 (http://evolve.zoo.ox.ac.uk/software.html?id=tracer) to ensure that stationarity was achieved after the first 200 K generations by checking whether the log-likelihood values of the sample points reached a stable equilibrium value (Huelsenbeck & Ronquist 2001). Of the remaining 2×18 K trees a majority rule consensus tree with average branch lengths was calculated using the sumt option of MrBayes. PPs were obtained for each clade. Clades receiving BS support equal or above 70 % under MP, and PPs equal or above 0.95 were considered strongly supported. Phylogenetic trees were visualized using the program Treeview (Page 1996).

Results and discussion

We generated 17 PKS sequences from 15 lichenized ascomycetes using the primer combination LC1/ISATc. Two paralogues each were amplified in *Pertusaria amara* and *P. tetrathalamia*. Four new sequences from four different *Pertusaria* species were obtained using the primer combination LC3/BERTc. Sequence lengths ranged from 1596 in *P. amara* (1066q) to 1715 in *P. pustulata* (1625f). *P. pustulata* (1625f) contained a 110 bp intron, and *P. pertusa* (1370q), *P. plittiana* (1511q) and *P. tetrathalamia* (1508q) each had two smaller introns (48–51 bp). All introns showed the characteristic GT-intron-AG splice sites.

The alignment of 112 sequences included 536 amino acid positions, 521 of which were variable and 509 were parsimony informative. The MP analysis yielded two most parsimonious trees, which were combined into a strict consensus tree (RI = 0.57, CI = 0.30) (tree not shown). Upon convergence of the MCMC procedure during the Bayesian analysis the WAG model of amino acid substitution (Whelan & Goldman 2001) contributed most significantly to the analysis (PP 1.00). The tree topologies obtained from MP and Bayesian analyses were almost identical, and hence only the phylogenetic tree of the Bayesian analysis is presented.

Fig 2 shows the 50 % majority-rule consensus tree of the Bayesian analysis. Fungal PKS genes fall into three well-supported groups (clades 1–3). Sequences in clade 1 belong to non-reducing PKS showing the minimal domain architecture KS-AT-ACP, plus one or two optional ACP and an optional CYC domain. The upper part of Clade 1, supported by PP 0.95 and BS 71 %, corresponds to 'non-reducing PKS clade I' in Kroken et al. (2003). It includes PKS genes coding for fungal toxins, such as sterigmatocystin, aflatoxin (i.e. their norsolorinic acid precursor), cercosporin, and dothistromin, and also for red, yellow, and brown pigments, e.g. naphtopyrone and aurofusarin, which play a role as virulence factors (Langfelder et al. 1998). Additionally, this group contains several paralogous PKS genes from lichenized fungi, corresponding to clades I-a, I-b, I-c, I-d, and I-f in Schmitt et al. (2005). *P. plittiana* (1511z) and *P. tetrathalamia* (1508z) probably represent a new PKS paralogue. Further characterized genes in clade 1 include those involved in melanin formation through oxidation/polymerization of di- (DHN) or tetrahydroxynaphtalene (THN) via the pentaketide pathway (Langfelder et al. 1998; Plonka & Grabacka 2006). The functions of all other PKS groups in this clade, including a further lichen-group [*P. pertusa* (1370q), *P. plittiana* (1511q) and *P. tetrathalamia* (1508q), corresponding to '*P.s.str.* clade XI' in Schmitt et al. (2005)], are unknown. This result shows that the combination of the degenerate primers LC1/ISATc amplifies multiple paralogous non-reducing PKS genes from lichen mycobionts. Most of them are related to PKS genes responsible for toxin and pigment biosynthesis, whereas one paralogue, represented by *P. pertusa*, *P. plittiana*, and *P. tetrathalamia*, forms an independent branch and is not closely related to any PKS gene with known function. Clade 2 contains non-reducing PKSs featuring a methyl transferase domain (ME). It corresponds to 'non-reducing PKS clade III' in the study of Kroken et al. (2003), and shows the domain configuration KS-AT-ACP-(ACP)-ME-(CYC). The only characterized gene in this clade is pksCT of *Monascus purpureus* involved in citrinin production. We expect that lichenized fungi also possess PKS genes that fall into this clade; however, the primer combination LC1/ISATc used in this study failed to amplify any of these. Clade 3 is composed of partially reducing PKS genes involved in 6-MSA and ochratoxin biosynthesis. Complete PKS genes in this group have the domain order KS-AT-DH-KR-ACP. The primer combination LC3/BERTc succeeded in amplifying PKS genes of this group also in lichenized fungi. Supported sister group to this clade of fungal 6-MSAS-type PKS is an assemblage of four bacterial type I PKS coding for the biosynthesis of orsellinic acid, 6-MSA, and naphtoic acid. The PKS-NRPS (PKS24) of *Cochliobolus heterostrophus* is an outlier among the fungal PKS genes and tends to form an unsupported relationship with bacterial PKSs (see also Kroken et al. 2003). The overall phylogeny using the extended alignment consisting of KS + linker + partial AT region is very similar to the phylogeny based on KS domain alone (Kroken et al. 2003). This finding corroborates the usefulness of the conservative KS region for phylogenetic analyses.

Genome searches of nine genomes revealed that reducing and non-reducing PKS genes are unevenly distributed across fungal genomes. Although 50 % of the detected PKS sequences in *Aspergillus nidulans* belong to the non-reducing type, only *ca* 15 % are non-reducing in *Chaetomium globosum*, *Gibberella moniliformis* and *Neurospora crassa* (Table 2). Of these nine genomes only *Aspergillus terreus* and *Cochliobolus heterostrophus* contain 6-MSAS-type genes. Our results demonstrate that the new primer combinations are useful in detecting mycobiont PKS genes potentially involved in the biosynthesis of typical lichen compounds. For the first time we show that lichenized fungi possess PKS genes of the 6-MSAS-type. Their role in the biosynthesis of metabolites remains to be studied. The retrieval of a PKS fragment spanning KS, linker and AT region will facilitate future primer design, and probe development for screening DNA libraries. Phylogenetic analysis using elongated sequence alignments supports the overall topology of major groups of PKS genes (Kroken *et al.* 2003; Schmitt *et al.* 2005).

Acknowledgements

We thank Yoshikazu Yamamoto for providing mycobiont cultures, Armin Mangold for conducting chemical analyses, and Gerald Lackner for bioinformatic support. This study was supported by a fellowship within the Emmy Noether-Program of the Deutsche Forschungsgemeinschaft to I.S., a Field Museum internship to S.K., and a start-up fund from the Field Museum to H.T.L. DNA sequences were generated in the Pritzker Laboratory for Molecular Systematics and Evolution at the Field Museum (Chicago).

REFERENCES

Ahlert J, Shepard E, Lomovskaya N, Zazopoulos E, Staffa A, Bachmann BO, Huang K, Fonstein L, Czisny A, Whitwam RE, Farnet CM, Thorson JS, 2002. The calicheamicin gene cluster and its iterative type I enediyne PKS. *Science* **297**: 1173–1176.

Archer A, 1993. A chemical and morphological arrangement of the lichen genus *Pertusaria*. *Bibliotheca Lichenologica* **53**: 1–17.

Beck J, Ripka S, Siegner A, Schiltz E, Schweizer E, 1990. The multifunctional 6-methylsalicylic acid synthase gene of *Penicillium patulum*. Its gene structure relative to that of other polyketide synthases. *European Journal of Biochemistry* **192**: 487–498.

Bingle LEH, Simpson TJ, Lazarus CM, 1999. Ketosynthase domain probes identify two subclasses of fungal polyketide synthase genes. *Fungal Genetics and Biology* **26**: 209–223.

Brodo E, 1991. Studies in the lichen genus *Ochrolechia*. 2. Corticolous species of North America. *Canadian Journal of Botany* **69**: 733–772.

Brown DW, Adams TH, Keller NP, 1996. *Aspergillus* has distinct fatty acid synthases for primary and secondary metabolism. *Proceedings of the National Academy of Sciences, USA* **93**: 14873–14877.

Culberson CF, 1964. Joint occurrence of a lichen depsidone and its probable depside precursor. *Science* **143**: 255–256.

Culberson WL, Culberson CF, 1970. A phylogenetic view of chemical evolution in the lichens. *Bryologist* **73**: 1–31.

Culberson WL, Culberson CF, 1977. Chemosyndromic variation in lichens. *Systematic Botany* **1**: 325–339.

Dayan FE, Romagni JG, 2001. Lichens as a potential source of pesticides. *Pesticide Outlook* **6**: 229–232.

Dibben M, 1980. The chemosystematics of the lichen genus *Pertusaria* in North America north of Mexico. *Publications in Biology and Geology, Milwaukee Public Museum* **5**: 1–162.

Elix JA, Jenie UA, Parker JL, 1987. A novel synthesis of the lichen depsidones divaronic acid and stenosporonic acid, and the biosynthetic implications. *Australian Journal of Chemistry* **40**: 1451–1464.

Felsenstein J, 1985. Confidence limits on phylogenies: an approach using the bootstrap. *Evolution* **39**: 783–791.

Grube M, Blaha J, 2003. On the phylogeny of some polyketide synthase genes in the lichenized genus *Lecanora*. *Mycological Research* **107**: 1419–1426.

Hanko B, 1983. Die Chemotypen der Flechtengattung *Pertusaria* in Europa. *Bibliotheca Lichenologica* **19**: 1–297.

Hanko B, Leuckert C, Ahti T, 1985. Beiträge zur Chemotaxonomie der Gattung *Ochrolechia (Lichenes)* in Europa. *Nova Hedwigia* **42**: 165–199.

Hawksworth DL, 1976. Lichen chemotaxonomy. In: Brown DH, Hawksworth DL, Bailey RH (eds), *Lichenology: progress and problems*. Academic Press, London, pp. 139–184.

Huelsenbeck JP, Ronquist F, 2001. MrBAYES: Bayesian inference of phylogenetic trees. *Bioinformatics* **17**: 754–755.

Huelsenbeck JP, Ronquist F, Nielsen R, Bollback JP, 2001. Bayesian inference of phylogeny and its impact on evolutionary biology. *Science* **294**: 2310–2314.

Huneck S, 2002. Die wasserabweisende Eigenschaft von Flechtenstoffen. *Bibliotheca Lichenologica* **86**: 9–12.

Jia X-Y, Tian Z-H, Shao L, Qu X-D, Zhao Q-F, Tang J, Tang G-L, Liu W, 2006. Genetic characterization of the chlorothricin gene cluster as a model for spirotetronate antibiotic biosynthesis. *Chemistry and Biology* **13**: 575–585.

Karplus K, Barrett C, Hughey R, 1998. Hidden Markov models for detecting remote protein homologies. *Bioinformatics* **14**: 846–856.

Kroken S, Glass NL, Taylor JW, Yoder OC, Turgeon BG, 2003. Phylogenomic analysis of type I polyketide synthase genes in pathogenic and saprobic ascomycetes. *Proceedings of the National Academy of Sciences, USA* **100**: 15670–15675.

Lange OL, Green TGA, Reichenberger H, Hesbacher S, Proksch P, 1997. Do secondary substances in the thallus of a lichen promote CO_2 diffusion and prevent depression of net photosynthesis at high water content? *Oecologia* **112**: 1–3.

Langfelder K, Jahn B, Gehringer H, Schmidt A, Wanner G, Brakhage AA, 1998. Identification of a polyketide synthase gene pksP of *Aspergillus fumigatus* involved in conidial pigment biosynthesis and virulence. *Medical Microbiology and Immunology* **187**: 79–89.

Larget B, Simon DL, 1999. Markov chain Monte Carlo algorithms for the Bayesian analysis of phylogenetic trees. *Molecular Biology and Evolution* **16**: 750–759.

Lumbsch HT, 1998a. The use of metabolic data in lichenology at the species and subspecific levels. *Lichenologist* **30**: 357–367.

Lumbsch HT, 1998b. Taxonomic use of metabolic data in lichen-forming fungi. In: Frisvad JC, Bridge PD, Arora DK (eds), *Chemical Fungal Taxonomy*. Marcel Dekker, New York, pp. 345–387.

Lumbsch HT, Schmitt I, Barker D, Pagel M, 2006. Evolution of micromorphological and chemical characters in the lichen-forming fungal family *Pertusariaceae*. *Biological Journal of the Linnean Society* **89**: 615–626.

Miao V, Coeffet-LeGal M-F, Brown D, Sinnemann S, Donaldson G, Davies J, 2001. Genetic approaches to harvesting lichen products. *Trends in Biotechnology* **19**: 349–355.

Nicholson TP, Rudd BAM, Dawson M, Lazarus CM, Simpson TJ, Cox RJ, 2001. Design and utility of oligonucleotide gene probes for fungal polyketide synthases. *Chemistry and Biology* **8**: 157–178.

Page RDM, 1996. Treeview: an application to display phylogenetic trees on personal computers. *Computer Applications in the Biosciences* **12**: 357–358.

Plonka PM, Grabacka M, 2006. Melanin synthesis in microorganisms — biotechnological and medical aspects. *Acta Biochimica Polonica* **53**: 429–443.

Sauer M, Lu P, Sangari R, Kennedy S, Polishook J, Bills G, An Z, 2002. Estimating polyketide metabolic potential among non-sporulating fungal endophytes of *Vaccinium macrocarpon*. *Mycological Research* **106**: 460–470.

Schmitt I, Lumbsch HT, 2004. Molecular phylogeny of the *Pertusariaceae* supports secondary chemistry as an important systematic character set in lichen-forming ascomycetes. *Molecular Phylogenetics and Evolution* **33**: 43–55.

Schmitt I, Martin MP, Kautz S, Lumbsch HT, 2005. Diversity of non-reducing polyketide synthase genes in the *Pertusariales* (lichenized *Ascomycota*): a phylogenetic perspective. *Phytochemistry* **66**: 1241–1253.

Schmitt I, Yamamoto Y, Lumbsch HT, 2006. Phylogeny of *Pertusariales (Ascomycotina)*: resurrection of *Ochrolechiaceae* and new circumscription of *Megasporaceae*. *Journal of the Hattori Botanical Laboratory* **100**: 753–764.

Souza-Egipsy V, Valladares F, Ascaro C, 2000. Water distribution in foliose lichen species: interactions between method of hydration, lichen substances and thallus anatomy. *Annals of Botany* **86**: 595–601.

Staunton J, Weissmann K, 2001. Polyketide biosynthesis: a millennium review. *Natural Product Reports* **18**: 380–416.

Swofford DL, 2003. *PAUP*: phylogenetic analysis using parsimony (*and other methods)* Version 4. Sinauer Associates, Sunderland, MA.

Weitnauer G, Mühlenweg A, Trefzer A, Hoffmeister D, Süssmuth RD, Jung G, Welzel K, Vente A, Girreser U, Bechthold A, 2001. Biosynthesis of the orthosomycin antibiotic avilamycin A: deductions from the molecular analysis of the *avi* biosynthetic gene cluster of *Streptomyces viridochromogenes* Tü57 and production of new antibiotics. *Chemistry and Biology* **8**: 569–581.

Whelan S, Goldman N, 2001. A general empirical model of protein evolution derived from multiple protein families using a maximum-likelihood approach. *Molelular Biolology and Evolution* **18**: 691–699.

Yamamoto Y, Kinoshita Y, Yoshimura I, 2002. Culture of thallus fragments and redifferentiation of lichens. In: Kranner I, Beckett RP, Varma AK (eds), *Protocols in Lichenology: culturing, biochemistry, ecophysiology and use in biomonitoring*. Springer–Verlag, Berlin, pp. 34–46.