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Diversity of Type I Polyketide Synthase Genes in a Bioactive *Phoma sp.* Endophytic Fungus isolated from *Cinnamomum molissimum* in Malaysia

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Endophytic fungi are known as excellent producers of bioactive natural products. An endophytic *Phoma* sp. fungus isolated from the medicinal plant *Cinnamomum molissimum* in Malaysia was found to produce several polyketide compounds including 5-hydroxyramulosin, a potent antifungal and cytotoxic compound with unknown biosynthetic pathways. The diversity of type I polyketide synthase (PKS) genes in this fungus was evaluated with a PCR-based approach using eight degenerate primer pairs targeting the ketosynthase (KS) and ketosynthase-acyltransferase (KS-AT) interdomain. Following amplification, 11 distinct putative PKS gene fragments were cloned and sequenced. Phylogenetic analysis of the PKS amino acid sequences revealed the biosynthesis potential for a wide range of polyketide compounds, including reduced, partially reduced and non-reduced polyketides. The PKS genes identified include one for the synthesis of tetrahydroxynaphthalene, and two apparently novel genes which differ from presently known PKS genes. These results demonstrate that PKS genes in the endophytic bioactive *Phoma* sp. are diverse and this isolate has the genomic capacity for producing more than ten different polyketide compounds including possibly unique compounds. The degenerate PCR approach was found to be a valuable method for determining the diversity of PKS genes and could be used to screen fungi with potential for producing bioactive compounds.

Key words: Endophytic fungi, natural products, PKS genes, *Phoma* sp., *Cinnamomum mollissimum*, degenerate primers.

Natural products are an important source of drugs since ancient times and about half of the useful drugs today are derived from natural sources [1]. Most drugs from natural products are actually plant and microbial secondary metabolites that have no apparent role in primary metabolism or growth. Endophytic fungi are fungal microorganisms which asymptomatically inhabit plant tissues and are capable of producing a variety of secondary metabolites [2, 3]. Apart from providing survival value to the plant, these metabolites may also have the potential for use in modern medicine[4]. The most abundantly produced fungal secondary metabolites are polyketide compounds [5].

Polyketide (PK) compounds possess a chain of alternating ketone and methylene groups [6]. Biological activities associated with polyketide metabolites encompass antibacterial, antiviral, antitumor, antihypertensive, anti-insect,

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immunosuppressant as well as mycotoxic activity as seen in the highly carcinogenic aflatoxin[7]. Early chemical and biochemical approaches established the relationship between polyketide and fatty acid biosynthesis, in which the carbon backbones of the molecules are assembled by successive condensation of small acyl units. The assembly process is controlled by multifunctional enzyme complexes called PKS, where the short chain carboxylic acids, usually acetyl coenzyme A (acetyl CoA) and malonyl CoA are condensed to form carbon chains of varying lengths [4, 8]. The discrete enzymes responsible for the individual loading, condensation, reducing, and chain-termination steps are referred to as domains or modules. When these catalytic domains are housed on a single massive polypeptide (often> 200kDa in size) the PKS is called a type I system [9].

Thus far, it has been found that fungal PKSs are iterative Type I enzymes. The type I iterative PKS consists of a single structural module, which is used repeatedly (iteratively) and therefore can be considered as multiple functional modules [10, 11]. The simplest functional PKS consists of a KS (β-ketoacyl synthase), an AT (acyltransferase), an ACP (acyl carrier protein) and a TE (thioesterase) domain. Based on domain organization, PKSs are divided into three groups; 6-Methylsalicylic acid synthase/ orsellinic acid synthase type (MSAS/ OAS) (partially reduced), aromatic multi-ring PKS (non reduced or WA type), and reduced complex type PKS (RD-PKS) [12, 13] (Table 1). Genes responsible for producing PKS have highly conserved domains, most notably within the KS domain [12]. It has been shown possible to speculate on the domain structure of the entire protein based on the KS sequence alone [11, 21], and therefore to speculate on the structure of the polyketide product itself. This information will allow researchers to identify and determine which putative PKS genes might be most interesting with respect to their potential to produce novel biologically active compounds [22].

A *Phoma* species fungus (CB007WA) was isolated from the traditional medicinal plant, *Cinnamomum molissimum,* in Bangi Forest Reserve, Malaysia. Species of the anamorph genus *Phoma* are commonly isolated from a wide range of ecological niches [23]. They are also one of the most frequent taxa isolated as endophytic fungi [24, 25]. *Phoma* sp. CB007WA was an interesting endophytic fungus because of its unique morphological features and its bioactive potential. This isolate may be a new species due to its unreported asexual structure in culture compared to known asexual forms and its DNA sequence that does not match with other identified fungal sequences. Identification of bioactive compounds in *Phoma* sp. CB007WA extract revealed the presence of at least three polyketide compounds including 5-hydroxyramulosin, which was the major polyketide compound produced, possessing potent antimicrobial and cytotoxic activity[26]. Since fungal polyketides play an important role in drug discovery, this study aimed to determine the diversity of PKS genes in the *Phoma* sp. CB007WA. Different sets of previously designed degenerate primers targeting ketosynthase (KS) and ketosynthase-acyltransferase interdomain (KS-AT) regions were used in PCR amplification followed by cloning, sequencing and phylogenetic analysis of PKS gene fragments.

MATERIALS AND METHODS

Fungal strain, culture conditions and genomic DNA isolation

CB007WA was isolated in Malaysia from the plant *Cinnamomum mollissimum* as an endophytic fungus and is deposited in the CBS[1] culture collection (CBS 133429). The isolate was cultured in 300 ml potato dextrose broth (PDB) and incubated at room temperature for 2-3 weeks. The mycelia, separated from the broth, were frozen and crushed using a pestle and mortar with CTAB lysis buffer and sterile sand. Mycelial lysate was extracted with phenol-chloroform-isoamyl alcohol. The fungal DNA was ethanol precipitated and resuspended in water for use in PCR.

PCR amplification of PKS genes and cloning of purified PCR products

PKS genes were amplified from the genomic DNA using various sets of previously designed degenerate primers for KS and KS-AT interdomain regions. Primer pairs KAF1, KAF2 / KAR1, KAR2, XKS1/XKS2, KS3/KS4, LC1/LC2 and LC3/LC5 were used in this study (Table 2). PCR was performed in a 50µL reaction volume with 1µg genomic DNA, 1×Hotstart Taq™ buffer (Qiagen, Hilden, Germany), 2.5mM $MgCl_2$, 0.2mM

of each dNTP, 0.6 µM of each primer, and 1.5 U HotStarTaq™ DNA polymerase (Qiagen). The thermal cycling program was 15min at 95°C followed by 35 cycles of 0.5 min at 94°C, 1 min at 51.7°C, and 2min at 72°C plus 7 min final extension at 72°C. All PCR products were run on a 0.8% agarose gel for better separation of multiple PCR products. To clone the amplified DNA, the desired bands were cut from the gel using a clean scalpel under weak UV light and purified using QIAquick gel extraction kit (Qiagen, Hilden, Germany). The expected size of PCR products from KA (ketosynthase- acyltransferase) and LC series primers was 700-800 bp while the XKS series produce 1.1-1.2 kb PCR fragments. The expected size of amplicons from KS series primers was 700-750 bp.

DNA cloning of purified PCR products was performed using pGEM®-T Easy vector systems (Promega, USA) followed by transformation of plasmid DNA into *Escherichia coli* strain JM109. The positive clones were identified on LB/ampicillin/IPTG/X-Gal agar plates and confirmed by colony PCR to have the plasmid insert following which plasmid isolation was performed.

Plasmid DNA isolation, restriction enzyme analysis, and DNA sequencing of cloned PKS gene fragments

A single colony from a freshly streaked LB/ampicillin agar was inoculated into 5mL of LB/ampicillin broth (100µg ampicillin/ml) and incubated for 12-16 hours at 37°C. The bacteria were harvested by centrifugation at 3000 x g for 10 min at 4°C. Isolation of recombinant plasmid from harvested bacteria was performed using QIAprep spin Miniprep kit (Qiagen, Hilden, Germany).

To differentiate between two cloned DNA fragments of the same size but with different sequences, restriction analysis was performed on 1 µl of plasmid preparations (0.5-1 µg/ µl). EcoR1 (Fermentas, Europe) was used for single digestion based on the pGEM®-T Easy vector map. For double digestion, three sets of restriction enzymes; (*EcoRI, StyI*), (*StyI, PstI*) and (*EcoRI, HphI*) (Fermentas, all in tango buffer®) were selected using NEB cutter (V2.0) [29] or restriction mapperV3 (http://www.restrictionmapper.org) programs. After comparing the digestion patterns on agarose gels, purified plasmid preparations with distinct patterns were selected for sequencing from both ends using the universal sequencing primers, SP6 and T7 promoters.

Extending the PKS gene sequence by DNA walking technique

Genomic sequences of two PKS gene fragments initially amplified with LC1/LC2 primers were extended using the DNA Walking SpeedUp™ Kit (Seegene, Seoul, Korea). The DNA walking technique allowed amplification of unknown sequences adjacent to known sequences. The kit consisted of a PCR master mix and unique DNA Walking Annealing Control Primers (DW-ACP). The target specific primers (TSPs) were designed from the cloned fragment sequences by using Primer3 software (V. 0.4.0) and purchased from 1st Base, Malaysia.

All PKS gene fragments were submitted to GenBank to obtain accession numbers. The accession numbers of PKS gene fragments for 111,121, 211, 225, 3, 4, 71, 72, 74, 81, and 82 are JQ621862, JQ621863, JQ621864, JQ621865, JQ621866, JQ621867, JQ621868, JQ621869, JQ621870, JQ621871, and JQ621872 respectively.

Phylogenetic analysis of PKS gene fragments

A BLASTx search was used to search for closest matched sequences in the GenBank database (http://www.ncbi.nlm.nih.gov) using cloned fragments' nucleotide sequences. Putative introns in DNA sequences were predicted by identifying the presence of GT/AG dinucleotides. After excising the putative introns, the deduced DNA sequences were subjected to BLASTx search again and submitted to GenBank databases.

To create a phylogenetic tree based on amino acid sequences, all retrieved DNA sequences were translated into amino acid sequences using Genetyx V. 3.1 (Japan 1996-1998). The deduced PKS amino acid sequences were aligned with KS-AT domain of the most similar published sequences from BLASTx results and selected reference sequences from GenBank database using CLUSTAL W [30]. The primer regions of all sequences were removed and not included in the alignment. For phylogenetic analysis, maximum parsimony bootstrap method [31] with heuristic search was performed using PAUP*version 4.0b10 [32] .The bootstrap analysis was set with 1000 replications, tree bisection-reconnection branch swapping, and random sequence addition. Gaps

were treated as missing data and given equal weight. The tree length, consistency indices (CI) and retention indices (RI) were calculated for each tree generated. The Kishino-Hasegawa (K-H) test was used for estimation of the best tree topology[33].

RESULTS

After PCR amplification of PKS genes using eight sets of degenerate primers and performing gel electrophoresis, ten distinct fragments were observed on the agarose gel (Fig. 1). For KA-series primers, KAF1/KAR1 and KAF1/KAR2 (lanes 1 and 2), two fragments each were amplified of 650-700 bp and 700-900 bp. The other KA-series primers, KAF2/KAR2 and KAF2/KAR1, amplified a single product of about 700 bp (lane 3 and 4). For the primer set XKS1/XKS2, a product of more than 1000 bp was observed (lane 5). KS3/KS4 primers did not amplify any product while LC1/LC2 (lane 7) amplified one distinct product of 700-850 bp and another of 900 bp. Primers LC3/LC5 amplified a 600-700 bp product (lane 8) (Table 3).

Following cloning and sequence analysis of PCR products, eleven putative PKS gene fragments were determined in CB007WA. Of these, six fragments were amplified by KA series primers (KAF1, KAF2/KAR1, KAR2) while five fragments were obtained using LC series degenerate primers (LC1/LC2, LC3/LC5) (Table 3). The XKS1/XKS2 and KS3/KS4 primer pairs did not detect any PKS genes. Two fragments (71 and 72) from lane 7 were extended successfully using DNA walking technique from 722bp and 771bp to 1762 and 1768bp respectively.

Table 1. Architecture and function of fungal PKSs

Type of fungal PKSs	Type of synthesized compounds	*Main functional domains	Examples of synthesized compounds
Methylsalicylic acid synthase /MSAS	Partially reduced	KS, AT, ACP,KR, DH	Patulin [14]
Polyketide synthases for aromatic multi-ring products (AR-PKSs) / (WA-type)	Non-reduced	KS, AT, ACP	Melanin [15], Aflatoxin [16]
Polyketide synthase for reduced products (RD-PKSs)	Reduced	KS, AT, ACP, KR, DH, ER	Lovastatin [17], citrinin [18], Fumonisin [19], T-toxin [20]

*KS: β-ketoacyl synthase, AT: acyltransferase, ACP: acyl carrier protein, and TE: thioesterase.

Table 2. List of primers used in the study [27]

Primer	Direction	Domain specificity	*Sequence(5'-3')
KAF1 [27]	Forward	KS	GAR KSI CAY GGI ACI GGI AC
KAF2 [27]	Forward	KS	GAR GCI CAY GCI ACI TCI AC
KAR1 [27]	Reverse	AT	CCA YTG IGC ICC RTG ICC IGA RAA
KAR2 [27]	Reverse	AT	CCA YTG IGC ICC YTG ICC IGT RAA
XKS1 [27]	Forward	KS	TTY GAY GCI BCI TTY TTY RA
XKS2 [27]	Reverse	KS	CRT TIG YIC CIC YDA AIC CAA A
KS3 [28]	Forward	KS	TTY GAY GCI GCI TTY TTY AA
KS4 [28]	Reverse	KS	RTG RTT IGG CAT IGT IAT ICC
LC1 [11]	Forward	KS	GAY CCI MGI TTY TTY AAY ATG
LC2 [11]	Reverse	KS	GTI CCI GTI CCR TGC ATY TC
LC3 [11]	Forward	KS	GCI GAR CAR ATG GAY CCI CA
LC5 [11]	Reverse	KS	GTI GAI GTI GCR TGI GCY TC

*B=C/G/T; D= A/G/T; I= Inosine; K=G/T; M= A/T; R= A/G; S=G/C; and Y= C/T

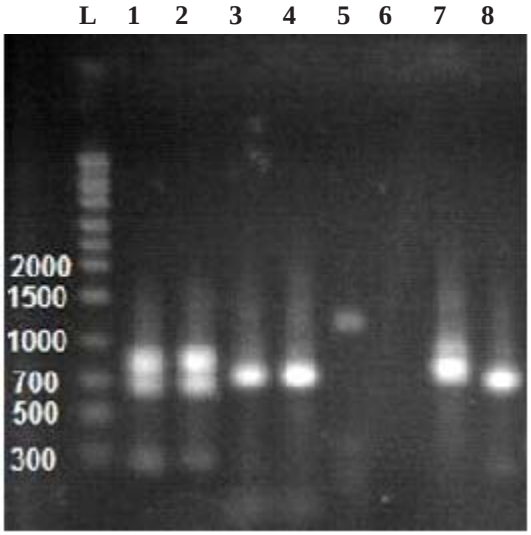
Table 3. Characterization of putative PKS genes identified in CB007WA

PKS gene fragment/ Gen Accession No.	Primer pair used	No. of putative introns/ size (bp)	PKS gene fragment length, without introns (bp)	The closest fungal PKS homolog based on BLASTx results/ GenBank Accession No.	Max identity (%)	E value
111/JQ621862	KAF1 / KAR1	-	675	Ketoacyl-synt-domain-containing protein [*Neurospora tetrasperma* FGSC 2509]/EGZ76371	52	8e-62
121/JQ621863	KAF1 / KAR1	5(68,27,15,30,15)	711	Polyketide synthase [*Trichoderma atroviride* IMI 206040]/ EHK50965	40	8e-42
211/JQ621864	KAF1 / KAR2	-	674	Putative Lovastatin nonaketide synthase [*Glarea lozoyensis* 74030]/ EHL02619	48	2e-51
225/JQ621865	KAF1 / KAR2	2(45,38)	708	Polyketide synthase [*Trichoderma atroviride* IMI 206040]/ EHK50965	41	8e-43
3/JQ621866	KAF2 / KAR2	-	717	Putative 6-MSAS-type polyketide synthase [*Pertusaria subfallens*] /ABQ11383	59	7e-88
4/JQ621867	KAF2 / KAR1	-	718	Putative 6-MSAS-type polyketide synthase [*Pertusaria subfallens*]/ ABQ11383	58	5e-81
71/JQ621868	LC1 / LC2	-	1762	1,3,6,8-tetrahydroxynaphthalene polyketide synthase protein [*Ascochyta rabiei*] /ACS74449	89	0.0
72/JQ621869	LC1 / LC2	2(52,24)	1692	Polyketide synthase [*Exophiala lecanii-corni*] /AAN74983	48	2e-162
74/JQ621870	LC1 / LC2	-	723	Putative non-reducing polyketide synthase [*Pertusaria hymenea*] AAY00101	96	6e-157
81/JQ621871	LC3 / LC5	-	566	Putative MSAS-type ketosynthase domain 2 [*Phoma* sp. C2932] /CAB44720	90	6e-119
82/JQ621872	LC3 / LC5	1(45)	649	Putative MSAS-type ketosynthase domain 2 [*Phoma* sp. C2932] /CAB44720	88	4e-130

Table 4. Diversity of PKS genes in CB007WA

Organism	PKS type		
	HR [a] PKS	NR [b] PKS	PR [c] PKS
Phoma sp. (CB007WA)	4	3	4

[a] Highly reducing, [b]Non-reducing, [c] partially reducing



L: 1 Kb DNA ladder. Primers used for amplification in Lane1: KAF1&KAR1, Lane2: KAF1&KAR2, Lane3: KAF2&KAR2, Lane4: KAF2&KAR1, Lane5: XKS1&XKS2, Lane6: KS3&KS4, Lane7: LC1&LC2, Lane8: LC3&LC5.

Fig. 1. PCR amplification of CB007WA PKS genes using eight sets of degenerate primers

The putative PKS gene sequences of CB007WA showed 41-96% identity to known fungal PKS gene sequences based on BLASTx results. The number of introns present was predicted to be between 0-5 (Table 3). A phylogenetic tree was constructed based on the deduced amino acid sequences. The eleven putative PKSs (ranging from 182-580 amino acids) aligned with deduced amino acid KS-AT domain sequences of 34 PKS genes and one *Homo sapiens* fatty acid synthase sequence as the out group. Out of 1065 characters, 107 were constant, 95 parsimony uninformative and 863 parsimony informative (tree length=11390, CI= 0.5810, RI= 0.5118) (Fig. 2).

DISCUSSION

Endophytic organisms have been proposed as an outstanding source of bioactive natural products [34]. Polyketides are a large family of structurally diverse natural products which possess a broad range of pharmacological properties that have gained widespread use in human medicine (as antibacterial, antifungal, and anticancer agents and immunosuppressants), veterinary medicine (as antibiotics and antihelmintics), and agriculture (as insecticides) [35]. Investigating the molecular basis of polyketide biosynthesis in fungi has importance for ecological and pharmacological practices [36]. As *Phoma* sp. CB007WA was known to produce polyketide compounds including a high level of 5-hydroxyramulosin [26], the diversity of its type I polyketide synthase genes was studied. This isolate was also of interest as it may be a new species due to its unique morphological characteristics and DNA sequences.

Using different sets of degenerate primers, eleven putative PKS gene fragments in CB007WA were amplified, cloned and sequenced. The number of PKS genes found in this fungus is similar to those of other fungi belonging to the subphylum Pezizomycotina (phylum Ascomycota) which is about 7-25 PKS genes [21]. The constructed phylogenetic tree based on KS-AT domains of the putative PKS enzymes (Fig. 2) showed very similar topology to previously established PKS gene analysis by Kroken *et al*. [21]. The CB007WA gene fragments placed in five different clades; non reducing PKS clade I, non reducing PKS clade II, 6MSAS, reducing clade IV and an unestablished clade. This tree demonstrated that CB007WA has different types of PKS genes and may be able to produce a wide range of polyketide compounds including highly reduced, non-reduced, and partially reduced polyketide compounds (Table 4).

Four CB007WA PKS fragments, 111, 121, 211 and 225 were amplified using KA series primers and placed among fungal reducing PKSs. It has been established that these primers are able to identify a variety of genes encoding PKS for highly reduced PK compounds [27]. Fragments 111 and 211 were nested in reducing PKS clade IV (Fig. 2). This clade includes *Gibberella moniliformis* FUM1 PKS which is responsible for the synthesis of PK precursor of the toxin fumonisin [37, 38]. Amino acid sequences 121 and 225 were placed in a new subclade, a sister group to reducing clade I. Their genes showed the least similarity (40-41%) to characterized PKS genes (Table 3). It is noted that the same unestablished clade was identified by Amnuaykanjanasin and co-workers [27] using the same primers. Thus it is probable that KA series primers are capable of identifying novel PKS genes. According to Kroken et al. [21], fungal non- reducing PKS clade includes enzymes that synthesize unreduced, and usually cyclic, i.e., aromatic, PKs that are precursors to toxins such as sterigmatocystin [39] and aflatoxin [16] or pigments such as melanin [15], bikaverin [40], and green spore pigments [41]. Three CB007WA PKS fragments, 71, 72, and 74 which were amplified using LC1/LC2

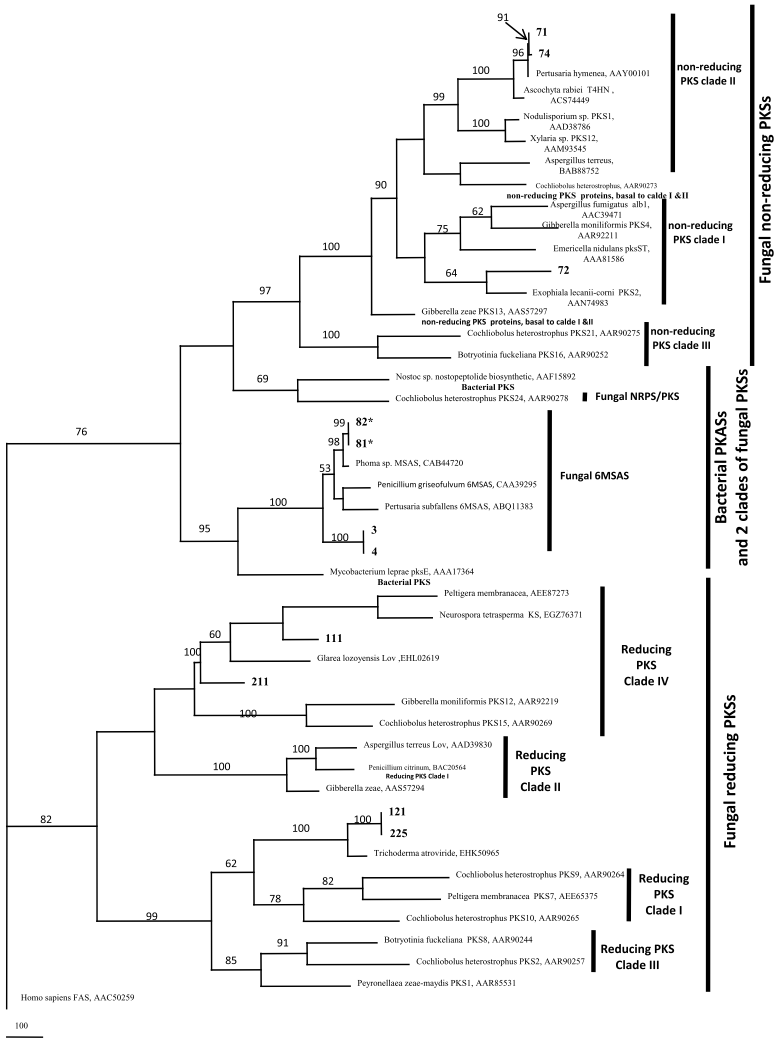


Fig. 2. Phylogram generated from parsimony analysis based on PKS amino acid sequence data; Bootstrap values ≤ 50% are shown above the branches, the tree is rooted with Homo sapiens fatty acid synthase (FAS), PKS gene fragments sequenced in this study (71, 74, 72, 81, 82, 3, 4, 111, 211, 121, 225) are printed in bold, bar = number of changes per nucleotide position.

primers were placed within this clade (Fig. 2). Amino acid sequence 71 and 74 showed high similarity to known PKS genes (Table 3). These sequences were grouped with *Pertusaria hymenea* polyketide synthase and *Ascochyta rabiei* tetrahydroxy naphthalene polyketide synthase proteins with high bootstrap support (100% BS) in a sister group to a non reduced PKS clade II characterized for melanin synthases. Sequence coded 72 showed 48% similarity to *Exophiala lecanii-corni* polyketide synthase and was grouped within non-reducing PKS clade I (Fig. 2).

Four fragments, 3, 4, 81, and 82 were nested within 6MSAS PKSs clade. The PKS of 6-methylsalicylic-type makes 6-methylsalicylic acid as a precursor to toxins such as patulin [14]. This fungal clade is placed in a large clade which includes all bacterial type I PKSs [21]. Fragment 3 and 4 were amplified using KAF2/KAR2 primers while PKS gene fragments 81 and 82 were obtained by LC3/LC4 primers.

In this experiment, cloning of PCR products from XKS1/XKS2 primers (band 5) was not successful. Improvement of techniques may lead to more successful cloning. Moreover, KS3/KS4 primers did not amplify any products in CB007WA. In a previous study that screened PKS genes [42], KS and FKS primers (a modified version of KS primers) were able to amplify PKS gene fragments in only 42% of fungal isolates studied. The results presented here demonstrate that the degenerate PCR approach has revealed the diversity of PKS genes in *Phoma* sp. CB007WA and the potential for producing different types of PK compounds including possibly unique compounds. The novel unestablished clade among reducing PKSs (Fig. 2), may refer to novel polyketide compounds of CB007WA. Duke et al. [43] suggested that fungi that possess unique PKS genes are more likely to produce unique and interesting PKS compounds. For further study, a genomic library could be made and fragments 121 and 225 can be used as probes for screening the library and obtaining the complete gene sequences. The complete sequence will help in the identification of interesting compounds and prediction of their functions. Next Generation Sequencing strategies, which have become more affordable, may also be used to obtain the complete organism genome sequences.

Analyses of complete fungal genome sequences have revealed a high number of PKS gene clusters in the fungi studied such as *Aspergillus flavus* (25 gene clusters) [44], however these may include silent genes which are not expressed or expressed at low levels. To determine which of the 11 PKS genes were actively expressed in the *Phoma* sp. CB007WA, primers listed in table 2 were used in PCR amplification of the fungal cDNA (obtained by fungal RNA extraction and cDNA synthesis, while ensuring genomic DNA was completely digested), which revealed 5 amplified gene fragments (111, 121, 211, 225, and 3) (results not shown) corresponding to the genome amplified fragments in lanes 1 to 3 in figure 1. Therefore, PCR amplification of cDNA confirms whether the polyketide products of interest (such as from unique PKS genes) are actually synthesised in the fungus. To induce expression of silent genes, a number of strategies have been investigated and this is an area of rapid research currently [45, 46].

In conclusion, the diversity of PKS genes in an endophytic fungus has been determined revealing the biosynthetic capacity for both unique and previously identified polyketide synthases. The degenerate PCR approach is a valuable method for understanding the diversity of PKS genes in fungi and could be used in screening of fungi with potential for producing bioactive compounds. This method may be especially useful to identify the biosynthetic capacity of novel fungal strains related to species which have known ecological or agricultural importance, such as entomopathogenic fungi.

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