CHAPTER 4

GENETIC STRATEGIES FOR SCREENING OF POLYKETIDE SYNTHASE GENES FROM SELECTED FUNGI

4.1 Introduction

Fungi are well known for their ability to produce a wide spectrum of chemical compounds. A major group of these metabolites, which have been the source of valuable chemotherapeutic agents, are the polyketides. The term polyketide was introduced in the literature in 1907, and secondary metabolites in 1891 (Benett and Bentley, 1989; Bentley and Benett, 1999). Polyketides are a large family of structurally diverse natural products found in plants, fungi and bacteria Fungi, particularly the anamorphic species, are a major source of polyketide metabolites (O'Hagan, 1991). The assembly of the initial carbon skeleton of a polyketide is catalysed by a large modular enzyme known as polyketide synthase (PKSs). PKSs are structurally similar to the fatty acid synthase enzymes and may share a common evolutionary origin (Hopwood and Khosla, 1992; Hutchinson and Fujii, 1995).

Recent literature on polyketide biosynthesis implies that PKSs have much greater diversity in both structure and mechanism than the current type I, II and III paradigms (Shen, 2003). Fungal PKSs are large multifunctional proteins (Type I PKS) encoded by a single complex gene possessing up to eight types of functional domain (Bingle *et al*., 1999). In contrast, the type II PKS enzymes found in bacteria are multi-enzyme complexes with the enzymatic domains carried on separate

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peptides. Type III PKS was first reported in 1999, are found in the actinomycetes (Funa *et al*., 1999; Shen, 2003).

Fungal polyketides present many unusual features not found in bacterial metabolites (O'Hagan, 1991). The diversity of fungal PKSs could be exploited in the generation of novel polyketides via combinatorial biosynthesis (Shimizu *et al*., 2005; Gaffoor *et al*., 2005; Bergmann *et al*., 2007) and newer sources of bioactive material like marine organisms (Smith *et al*., 2000; Gupte *et al*., 2002) and endophytic fungi (Strobel, 2006). Polyketide metabolites have been found in a large number of filamentous fungi (Fulton *et al*., 1999; Graziani *et al*., 2004). Particularly, useful and novel polyketides might be scaled up for industrial production and a world market (Chakravarti and Sahai, 2004; Shukla *et al*., 2005; Atoui *et al*., 2006).

Traditionally, the investigation of bioactive metabolites is started by growing a large number of fungi in fermentation broths, and tested the cultures (mycelium and medium filtrate) for bioactivities such as anti-microbial, anti-cancer and anti-malarial activities (Kang and Kim, 2004; Wiyakrutta *et al*., 2004). It seems that a vast supply of target bioactive compound remains to be discovered since we now know that fungi have many natural product gene clusters which are not expressed when the organism is grown in non-inducing conditions (Farnet and Zazopoulos, 2005). Many recently published papers have described a different approach which involves the use of degenerate PCR primers based sequences from identified *pks* genes to identify fungal strains that harbour these genes, and they have the potential to produce this group of metabolites (Várga *et al*., 2003; Grube and Blaha, 2003; Kroken *et al*., 2003; Amnuaykanjanasin *et al*., 2005). This approach could help to avoid the re-isolation of known compounds.

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The importance of drugs in the treatment of pathogenic microorganisms has led us to explore fungi with the potential to produce highly active bioactive compounds. Many of these have exploited as chemotherapeutic agents for human disease such as penicillin, erythromycin, bleomycin and lovastatin (Farnet and Zazopoulos, 2005). Fungal polyketides comprise a large group of structurally diverse secondary metabolites which have proved to be valuable lead molecules for the development of some of the most important drugs (Schümann and Hertweck, 2006) Fungal type I polyketides (PKs) are synthesized by multidomain enzymes using an iterative strategy to build up a polyketide (Fujii *et al*., 2001; Varga *et al*., 2003). The functional domains, namely ketosynthase (KS), acyl transferase (AT), acyl carrier protein (ACP) or phosphopantetheine (PP) are necessary for both FASs and PKSs. In addition, ketoreductase (KR) dehydrate (DH) enoyl reductase (ER) and thioesterase (TE) can be found in some PKSs and further optional accessory domains are functioned by methytransferase (MT) (Bingle *et al*., 1999) or cyclase (CYC) (Fujii *et al*., 2001). Fungal polyketides were divided into two subclasses using ketosynthase domain probes namely the non-reducing PKS (or WA) and reducing PKS (or MSAS) types (Bingle *et al*., 1999).

The synthesis of non-reducing PKSs involves chemical reduction in forming the structure of compounds. This group includes fungal pigments e.g. melanin, and mycotoxins such as aflatoxin. The synthesis of reduced PK compounds with various chemical reduction in structure is governed by reducing PKSs, with lovastatin, citrinin, fumonisin and T-toxin synthesis as examples of this group.

In this study, we have developed the primer pairs for the ketosynthase domain of reducing type I PKSs to identify partial PKS sequences from three novel fungi

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which responsible for reducing type I PK synthesis. These sequence data will be used as probes to isolate putative PKS genes in the further study. The purposes of the study are as following:

1. To study the diversity of *pks* genes from novel ascomycetes fungi.
2. To study the diversity of fungi related to *pks* genes and potential polyketide production.

4.2 Materials and methods

4.2.1 Fungal strains and genomic DNA isolation

The fungi used in this investigation are listed in appendix D (Bussaban, 2005; Promputtha, 2006; Bhilabutra, 2009). They were cultured in Potato Dextrose Broth (LabScan) and incubated at 30°C for 7 days. The mycelia were harvested and lyophilized, and stored at -80°C before DNA preparation. The DNeasy Plant Minikit (QIAGEN) was used for the preparation of genomic DNA.

4.2.2 Amplification of KS domains

PKS fragments were amplified using two primer pairs (ketosynthase domain specific primers): KAF1-KAR2 (Amnuaykanjanasin *et al*., 2005) and KSDIF-KSDIR (Fujii, personal communication). The standard PCR reaction was described in Table 4.1.

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Table 4.1 PCR reaction and thermal cycling condition

PCR Reagent	**Reaction Volume (µl)**	
	KA	**KSDI**
10X *Ex Taq* Buffer	2.5	2.5
dNTPs Mixture (2.5 mM each)	2.0	2.0
Template	< 500 ng	< 500 ng
Primer F	0.5 µM final conc.	0.01 pM final conc.
Primer R	0.5 µM final conc.	0.01 pM final conc.
TaKaRa Ex Taq™ (5 units/µl)	0.125	0.125
Distilled water	up to 25 µl	up to 25 µl
Thermal Cycling Condition	**Degenerate Primer**	
	KA	**KSDI**
Initial denaturation	95°C, 5 min	95°C, 12 min
Denaturation Annealing Extension	 94°C, 0.5 min 57°C, 1 min 72°C, 2 min × 30 cycles	 94°C, 0.5 min 50°C, 0.5 min 72°C, 1 min × 45 cycles
Final Extension	72°C, 7 min	72°C, 5 min

4.2.3 Cloning and sequencing of PCR fragments

The PCR fragments were cloned into pT7 Blue Vector using TaKaRa Mighty Mix (Takara, Japan). Competent cells of *E. coli* strain DH5α™ or XL 10 Gold™ were prepared for transformation (Strategene) as described in the suppliers handbook. Transformation was done according to the published protocol. Plates of LB agar, which contained kanamycin (50 µg/ml), were spread with 100 mM IPTG and 1% X-gal. Plates were incubated at 37ºC before use. Transformed cells were inoculated onto the agar and the cultures were incubated at 37ºC for 16-18 hrs. White colonies, which carried the inserted DNA fragments, were selected for PCR amplification. Positive

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colonies were sub cultured in LB broth, which contained kanamycin, and incubated on a rotary shaker at 37ºC, 180 rev.min^{-1} for 16 hrs. Bacterial cells were harvested for plasmid isolation using E.Z.N.A™ Plasmid Miniprep Kit (HiBind® technology, Omega Bio-tek, Inc). KS fragments were sequenced using M13 forward and reverse primers and BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) were used and applied with 3130 *xl* Genetic Analyzer (Applied Biosystems).

4.2.4 Sequence and phylogenetic analysis

Translated protein sequences were compared with those in the National Center for Biotechnology Information data base using BLASTx program (Altschul *et al*., 1990). PKS protein sequences were aligned using the PAM Dayhoff matrix and the multiple sequence alignment tool from CLUSTAL X 1.83 (Thompson *et al*., 1997) and MEGA package version 4.0 (Tamura *et al*., 2007). Significant bootstrap values were calculated from 1000 bootstrap re-samplings. Tree reconstruction was performed using the neighbor-joining method.

4.3 Results

4.3.1 Diversity of polyketide synthase from novel ascomycetes fungi

Polyketide synthases are responsible for the biosynthesis of many secondary metabolites in fungi. More recently PCR-based screening systems have proved to be an efficient means to identify organisms having the potential to produce polyketides (Amnuaykanjanasin *et al*., 2005; Gross *et al*., 2006; Barrios-Llerena *et al*., 2007; Amnuaykanjanasin *et al*., 2009).

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4.3.1.1 Amplification and analysis of KS domain sequences

Our newly designed primer KSDI (Fujii, personal communication) and the primer KA-series designed by Amnuaykanjanasin *et al*. (2005) were employed to identify the KS domain of PKSs in the genomes of the three fungi. These ketosynthase domain probes amplified target DNA fragments about 700-800 bp. Homology searches using BLASTp software (NCBI) were described in Table 4.2.

4.3.1.2 Clustering of fungal *pks* genes

Fungal type I PKSs are classified into three groups based on the metabolite types (Fujii *et al*., 2001; Várga *et al*., 2003) and the extent of reduction of the polyketide ring (Nicholson *et al*., 2001; Várga *et al*., 2003). Phylogenetic analysis based on the KS domain sequences enabled the identification of two subclasses: WA type and MSAS type (Funa *et al*., 2006). More recently, amino acid sequences of fungal KS domains have been used to identify four subclades based on the reduction of their products in reducing PKS (Kroken *et al*., 2003, Schümann and Hertweck, 2006).

We obtained 10 amplification products of KS sequences and based on phylogenetic analysis (Figure 4.1); these could be classified into three subclades based on the evolutionary relationships between different types of fungal KS domains (Kroken *et al*., 2003). The analysis data was not homologous to the reducing subclade I related to lovastatin/citrinin diketide. The reducing subclade II, which involved lovastatin/citrinin nonaketide, represented two unique KS domains from *L. amomi* and one unique KS domain from *G. amomi*. BLASTp analysis presented 52% identity to lovastatin nonaketide synthase of *P. brasiliensis* Pb03 (McEwen *et al*., unpublished).

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Table 4.2 Characterization of ten polyketide synthases identified from ascomycetes fungi

No.	Clone	Accession number	Primer Pair [a]	The closest PKS homolog (Identify of amino acid sequence, %) [b]	Accession number
1	B25KSDI1	ACR82777	KSDI	*Botryotinia fuckeliana*, polyketide synthase, 51% in 269 aa	AAR90247
2	B25KSDI2	ACR82778	KSDI	*Neosartorya fischeri* NRRL 181, polyketide synthase, 43% in 229 aa	EAW21094
3	B25KA41	ACR82776	KA	*Pyricularia longispora*, polyketide synthase,78% in 232 aa	AAR82776
4	B25KA5127	ACT53018	KA	*Glomerella* sp. EMMS3/35, polyketide synthase, 51% in 285 aa	ACT53021
5	B128KSDI2	ACR82779	KSDI	*Glomerella* sp. EMMS3/35, polyketide synthase, 95% in 267 aa	ACT53021
6	B128KSDI32	ACR82780	KSDI	*Paracoccidioides brasiliensis Pb03*, lovastatin nonaketide synthase, 52% in 249 aa	EEH16752
7	Lam4A	ACR82781	KA	*Periconia tirupatiensis* , polyketide synthase, 47% in 285 aa	ACR82785
8	LamK17	ACR82782	KSDI	*Aspergillus flavus* NRRL3357, putative polyketide synthase, 49%in 265 aa	EED51023
9	LamK63	ACR82783	KSDI	*Botryotinia fuckeliana*, polyketide synthase, 51% in 269 aa	AAR90247
10	LamK11	ACR82784	KSDI	*Kallichroma glabrum*, putative polyketide synthase, 56% in 232aa	ACN43280

[a] Degenerate primer pair used in the PCR amplification of the fragment.
[b] Homology searches using BLASTp software (NCBI). Amino acid sequences were deduced from the DNA sequences
[c] Abbreviation of clone: B25 (*Gaeumannomyces amomi*), B128 (*Leiosphearella amomi*), Lam (*Emarcea castanopsidicola*)

Leiosphearella amomi was cultivated in expression media for lovastatin production (Casas López *et al*., 2003, Chang *et al*., 2002, Sayyad *et al*., 2007), however, production of polyketides could not be detected. The one finding concerned the KS domain sequence isolated from *G. amomi* which was grouped with the reducing subclade III similar to putative polyketide synthase from *Sclerotinia sclerotiorum* 1980 and *B. fuckeliana pks8*. Another sequence from *G. amomi* was classified to the reducing subclade IV which corresponds to fumonisin synthesis. A unique KS domain from *E. castanopsidicola* was homologous to *K. glabrum* which is a marine fungus in tropical environments (Amnuaykanjanasin *et al*., 2009). Of the other, two KS domain fragments isolated from *E. castanopsidicola* showed homology to the putative polyketide synthase from *T. stipitatus* ATCC 10500 and *A. flavus* NRRL3357. Furthermore, we found that KS domain sequences from *E. castanopsidicola* and *G. amomi* were belonged to the same clade of *pks* from *B. fuckeliana* and *Penicillium marneffei* ATTC 18224. Homology of the two fragments indicated that they could probably be responsible for the synthesis of molecules in the same group of polyketides.

4.3.2 Diversity of fungi related to *pks* gene and potential PK production

Tropical countries are characterized by the availability and diversity of fungi, and other life forms, that are found in the range of terrestrial and aquatic habitats such as forests, freshwater reservoirs and seas. Uncountable numbers of species in Thailand have been found in different niches and habitats. The presence of KS domains of reducing type I polyketide synthase would lead to a prediction of possible polyketide production in selected fungi.

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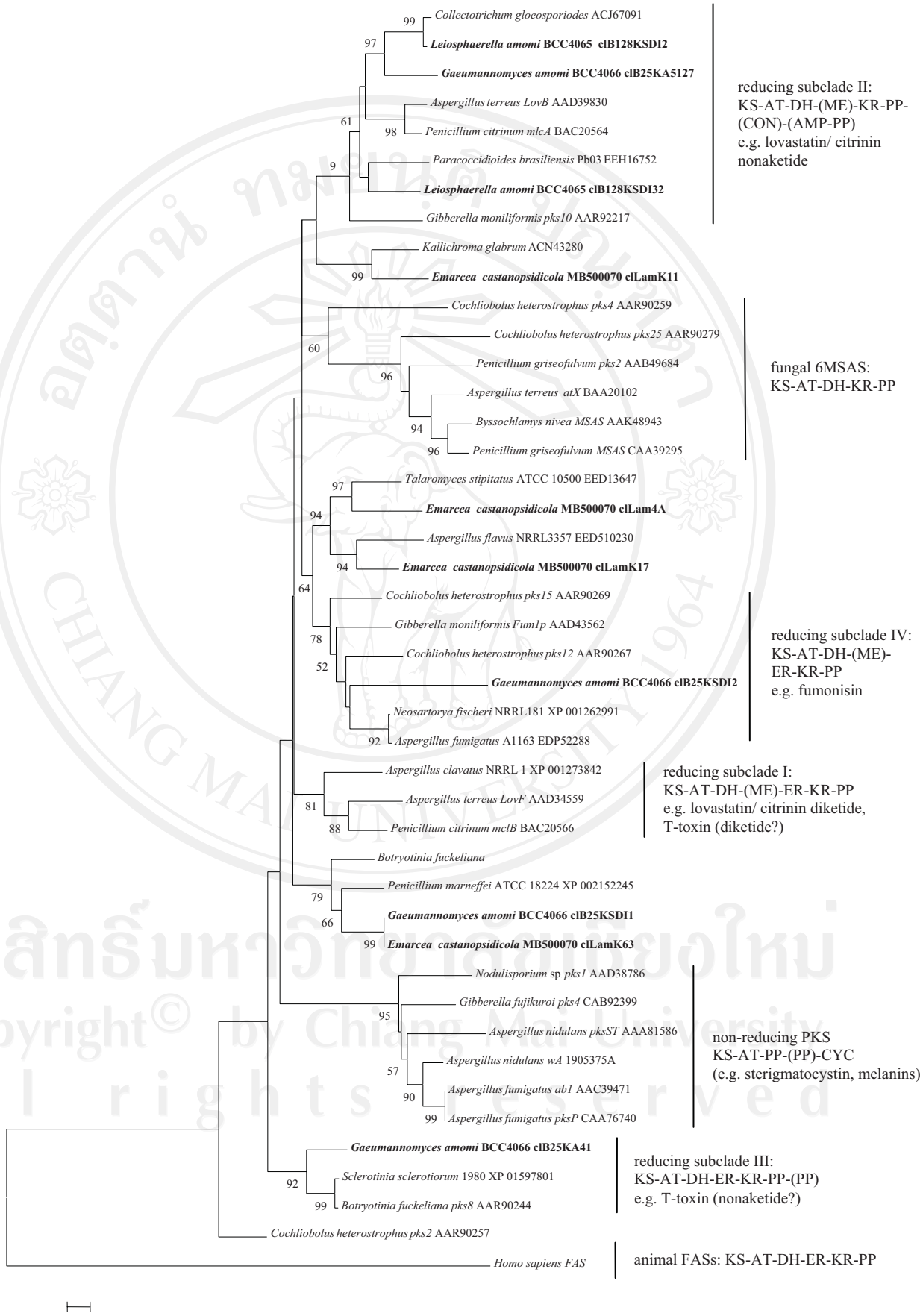

Figure 4.1 Neighbor-joining phylogenetic tree of deduced amino acid sequences of 10 fungal KS domain fragments and other known non-reducing and reducing fungal PKSs in the NCBI database. Bootstrap analysis using neighbor-joining was conduct with 1,000 replicates, and bootstrap values greater than 50% are given at node.

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4.3.2.1 PCR detection of *pks* gene fragments using KA-series primer

Twenty-three KS fragments were identified in our twenty selected fungi by PCR based screening. PCR products were cloned and sequenced; they showed high homologies to other PKSs according to BLASTp searches (Table 4.3-4.4).

4.3.2.2 KS-AT phylogeny and prediction of PK structure

For the KA (ketosynthase-acyltransferase)-series primers, forward primers (KAF1) were designed from the amino acid (aa) sequences in the conserved KS domain of reducing-type fungal PKSs, whereas the reverse primers (KAR2) were designed based on the sequences in the AT domain (Amnuaykanjanasin *et al*., 2005; Amnuaykanjanasin *et al*., 2009). Similarity search for the cloned DNA sequences were performed using BLASTX against the NCBI/GenBank database (). For phylogenetic analysis, the deduced amino acid sequences of all twenty-three PKS gene fragments identified in the selected species were aligned with other PKS amino acid sequences in the NCBI database. The resulting phylogram showed a similar clustering of reducing fungal PKSs subclade I-IV and non-reducing PKS, described in previously reported studies using KS domain sequences (Figure 4.2) (Bingle *et al*., 1999; Kroken *et al*., 2003; Amnuaykanjanasin *et al*., 2005). Three main clades of fungal PKSs were identified and correlated to structural classes of fungal PKs: reduced, partial reduced and unreduced type (Hopwood, 1997; Bingle *et al*., 1999).

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Table 4.3 Characterization of twelve polyketide synthases identified from tropical endophytic fungi

Taxa	Accession number	The closest PKS homolog (Identify of amino acid sequence, %)	Accession number
Endophytic fungi			
1. *Colletotrichum gloeosporioides* ZE0116	ACT53003	polyketide synthase *Leiosphaerella amomi*, 89% in 267 aa	ACR82779
2. *Colletotrichum* sp. EM5/6	ACT53009	polyketide synthase *Colletotrichum gloeosporioides*, 95 % in 267 aa	ACJ67091
3. *Cylindrocladium* sp. ZE0150	ACT53015	polyketide synthase *Gibberella moniliformis*, 71 % in 262 aa	AAR92213
4. *Eupenicillium crustaceum* ZE0151	ACT53008	polyketide synthase *Talaromyces flavus*, 99% in 240 aa	ACT53012
5. *Eupenicillium shearii*	ACT53014	polyketide synthase *Eupenicillium shearii*, 98% in 263 aa	ACR82790
Eupenicillium shearii	ACR82790	polyketide synthase *Eupenicillium shearii*, 98 % in 263 aa	ACT53014
6. *Fusarium* sp. EM2/23	ACT53005	polyketide synthase *Corollospora besarispora*, 58 % in 252	ABD47711
7. *Glomerella* sp. EMMS3/35	ACT53021	polyketide synthase *Leiosphaerella amomi*, 95 % in 267 aa	ACR82779
8. *Humicola fuscoatra* ZE0228	ACT53004	polyketide synthase, putative *Penicillium marneffei* ATCC 18224, 68% in 251 aa	EEA21220
9. *Pyricularia longispora* ZE0005	ACT53007	polyketide synthase *Gaeumannomyces amomi*, 78% in 232 aa	ACR82776
10. *Taralomyces flavus* ZE0199	ACT53012	polyketide synthase *Eupenicillium crustaceum*, 99 % in 240 aa	ACT53008
11. *Xylaria* sp. EMMS2/8	ACR82789	polyketide synthase, putative *Aspergillus flavus* NRRL3357, 41% in 295 aa	EED47890

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Table 4.4 Characterization of eleven polyketide synthases identified from tropical saprobic fungi

Taxa	Accession number	The closest PKS homolog (Identify of amino acid sequence, %)	Accession number
Saprobic fungi			
1. *Colletotrichum gloeosporioides* MSN050	ACT53016	polyketide synthase *Sagaaromyces glitra*, 67% in 239 aa	ACJ67106
2. *Calonectria kyotensis* MSN039	ACR82786	putative polyketide synthase *Halorosellinia oceanica* , 65% in 250 aa	ACN43267
3. *Dictyosporium digitatum*	ACR82787	polyketide synthase *Gibberella moniliformis*, 44% in 280 aa	AAR92209
Dictyosporium digitatum	ACR82788	putative polyketide synthase *Penicillium marneffei* ATCC 18224, 48% in 250 aa	EEA22389
4. *Hyponectria manglietiae* MS054	ACT53013	polyketide synthase *Nectria haematococca*, 99% in 272 aa	ACT53010
5. *Lasiodiplodia* sp. MSN026	ACT53011	polyketide synthase *Hypocrella discoidea*, 44% in 243 aa	ACJ67082
6. *Myrothecium pandanicola*	ACT53006	polyketide synthase *Gibberella moniliformis*, 50% in 262 aa	AAR92213
Myrothecium pandanicola	ACT53020	polyketide synthase *Botryotinia fuckeliana*, 51% in 236 aa	AAR90251
7. *Nectria haematococca* MSN045	ACT53010	polyketide synthase *Hyponectria* sp. JJ-2009a, 99% in 272 aa	ACT53013
8. *Nectria* sp. MSN047	ACT53017	polyketide synthase *Microsporum canis* CBS 113480, 58% in 238 aa	EEQ30372
9. *Periconia tirupatiensis* CMU26851	ACR82785	polyketide synthase *Monascus purpureus*, 98% in 272 aa	ACT53019

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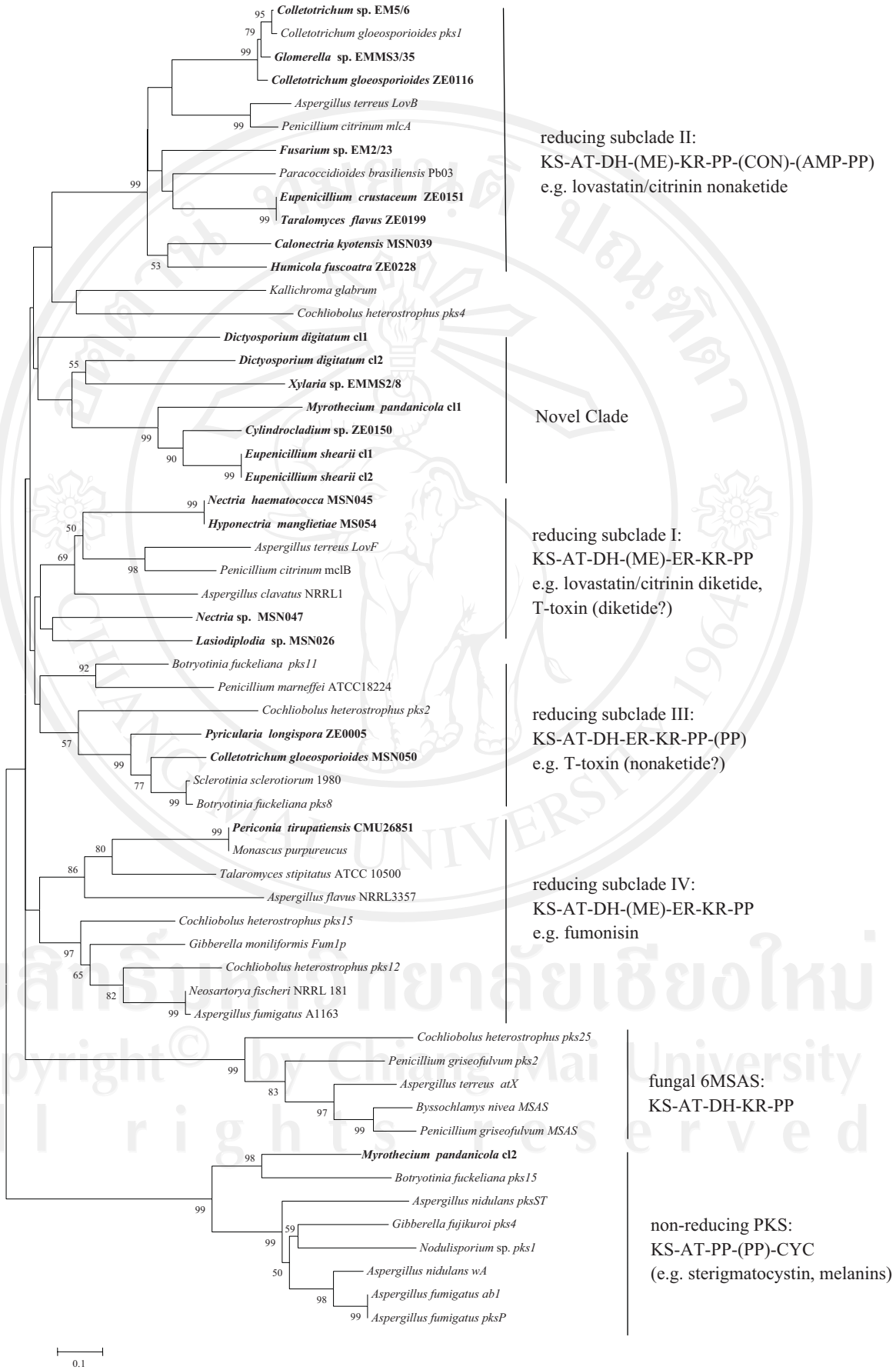

Figure 4.2 Neighbor joining phylogenetic tree of deduced amino acid sequences of 23 fungal KS domain fragments and other known reducing and non-reducing fungal PKSs in the NCBI database. Bootstrap analysis using NJ was conduct with 1,000 replicates, and values of ≥ 50% are given at node.

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Fungal PKSs producing Reduced PKs

The main fungal clade includes species that synthesize variously reduced types of PKs. The characterized PKs in this clade are lovastatin (Hendrickson *et al*., 1999), citrinin (Abe *et al*., 2002), T-toxin (Yang *et al*., 1996), PM toxin (Yoder, 1973) and fumonisin (Nelson *et al*., 1993). These molecule types are mostly synthesized from CoA thioesterified carboxylic acids other than acetyl and malonyl CoA. There are many predicted PKSs in this clade that are highly divergent (Kroken *et al*., 2003). The clade of reduced PKS was subdivided into four subclades (I-IV) (Figure 4.2).

Reducing PKS subclade I

The reducing PKS subclade I is characterized by the two compounds lovastatin and citrinin. Four isolates; *Nectria* sp. MSN047, *Lasiodiplodia* sp. MSN026, *Nectria haematococca* MSN045 and *Hyponectria manglietiae* MS054, were members of this subclade. In addition, the KS domain of *N. haematococca* MSN045 showed a very high similarity to *H. manglietiae* MS054, which may synthesize an homologous structural polyketide.

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Reducing PKS subclade II

Reducing PKS subclade II is characterized by the production of enzymes that have lost the ER domain and predicted to either contain alkyl groups whose reduction is completed by product of an external ER domain-containing gene for example *A. terreus lovC* (Figure 4.3) or lack a reduced alkyl group (Kroken *et al*., 2003). Furthermore, these functional enzymes were found either to include a condensation

(CON) domain typical of non- ribosomal peptide synthases (NPSs) or composed of a CON domain, an adenylation (AMP) domain, and a PP domain in whole NPS module.

Many of selected fungi; *Colletotrichum gloeosporioides* ZE0116, *Glomerella* sp. EMMS3/35, *Colletotrichum* sp. EM5/6, *Calonectria kyotensis* MSN039, *Humicola fuscoatra* ZE0228, *Eupenicillium crustaceum* ZE0151, *Taralomyces flavus* ZE0199 and *Fusarium* sp. EM2/23 were grouped into this subclade. The results show that KS domain of *E. crustaceum* ZE0151 is similar to that of *T. flavus* ZE0199, which may suggest that these fungi synthesize the ortholog portion of polyketide structures.

Reducing PKS subclade III

The gene sequence of the third reducing PKSs subclade appears to lack the ME domain in the enzyme system, but it had an additional PP domain. *Pyricularia oryzae* ZE0005 (Bussaban *et al*., 2003) and *C. gloeosporioides* MSN050 (Proputtha, 2006) fall into this group and are predicted to synthesize the same molecule type of compound. Nonetheless, only *Cochliobolus heterostrophus pks2* has been characterized and shown to synthesize T-toxin (Braker, personal communication).

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Reducing PKS subclade IV

PKSs in reducing subclade IV are similar to subclade I and II, in which a conserved ME domain may or may not be present. Analysis of the phylogram shows that the KS domain of *Periconia tirupatiensis* CMU26851 was homologous to *Monascus purpureus* CMU001.

Fungal PKSs Producing partial reduced and unreduced PKs

The other main clade of fungal PKS includes enzymes that synthesize unreduced PKs which usually have a cyclic form. Ketosynthase domain probes identified two subclasses, MSAS-type (partial reducing PKS, e.g. patulin and MSAS) and WA-type (non-reducing PKS, e.g. aflatoxin, sterigmatocystin, melanin), using LC1/LC2c and LC3/LC5c (Bingle *et al*., 1999). In this study, KA series primers were designed from the amino acid sequences in the conserved domain of reducing-type fungal PKSs but only one KS fragment from *M. pandanidicola* was grouped into subclade of non-reducing PKs. BLASTp analysis confirmed that KS fragment from *M. pandanidicola* presented 51% similarity in 236 aa of *Botryotinia fuckeliana pks15* (AAR90251). It is occasionally found that highly reduced type PKS designed primers can amplify any fragment of non-reduced PKS. The grouping of a second fragment to a reduced PKS cannot be explained based on the data obtained. *M. pandanicola* (Thongkantha et al., 2008) had weak antibacterial activity and elucidated a structure of 2,3-dihydro-5-methoxy-2-methylchromen-4-one which is an intermediate during polyketide synthesis pathway. Nevertheless, a presence of fragment in non-reducing clade may not be clearly explained by this study.

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Other Fungal PKSs

Seven KS domain sequences were found that could be divided merely by phylogenetic analysis. However, this subclade includes an uncharacterized functional enzyme system and it may suggest novel functions of these PKSs. Two KS domain sequences were found in *Dictyosporium digitatum* and an ortholog in *Eupenicillium shearii*. Moreover, *Cylindrocladium* sp. ZE0150, *Xylaria* sp.EMMS2/8 and

Myrothcium pandanicola were grouped into this clade. However, this should be confirmed by determination of PK products generated by these enzymes.

4.4 Discussion

4.4.1 Diversity of polyketide synthase from novel ascomycetes fungi

Ten KS domain sequences have been isolated using primer pairs specific for highly reduced type PKSs, which probably relate to putative PKS genes in these ascomycete fungi. Bioinformatics techniques were also employed to identify groups of these KS domain sequences and gene cluster genes of other enzymes involved in secondary metabolism and thus promising for investigation for possible lead molecules with potential for drug development. *G. amomi* and *L. amomi* were identified as novel endophytic fungi from *Amomum siamense* and have been previously evaluated for antibiotic production (Lumyong *et al*., 2004). It is now necessary to attempt to correlate the expression of KS containing clusters with the compounds produced by these fungi. This would confirm the importance of endophytic fungi as sources of polyketides, some of which may be of commercial value (Tan and Zou 2001, Strobel, 2006). For example, one fragment of a KS domain sequence from *L. amomi* showed 52% similarity to *Poccidioides brasiliensis Pb03*, lovastatin nonaketide synthase. The expression of the partial *pks* had been investigated by fermentation in many different media and using HPLC analysis (Chapter 5).

Previously, we isolated stemphol (Stodola *et al*., 1973; Marumo *et al*., 1985; Jumpathong *et al*., 2009) and stemphol 1-*O*-β-D-galactopyranoside, a novel stemphol derivative from *G. amomi* (Jumpathong *et al*., 2010). Stemphol was first isolated from

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Stemphylium majusculum (Stodola *et al*., 1973) and its related compounds had antimicrobial activities against *Mucor hiemalis*, yeast *Schizosaccharomyces pombe*, *Bacillus subtilis* and *Staphylococcus aureus* (Achenbach and Kohl, 1979). In 1985, stemphol was isolated from *Pleospora herbarum* and described as a self-inhibitor (Marumo *et al*., 1985). The stemphols were classified as phenolic or resorcinolic lipids (Figure 4.3) (Kozubek and Tyman, 1999) and these molecule types were synthesized by type III polyketide synthase (Funa *et al*., 2006, Funa *et al*., 2007).

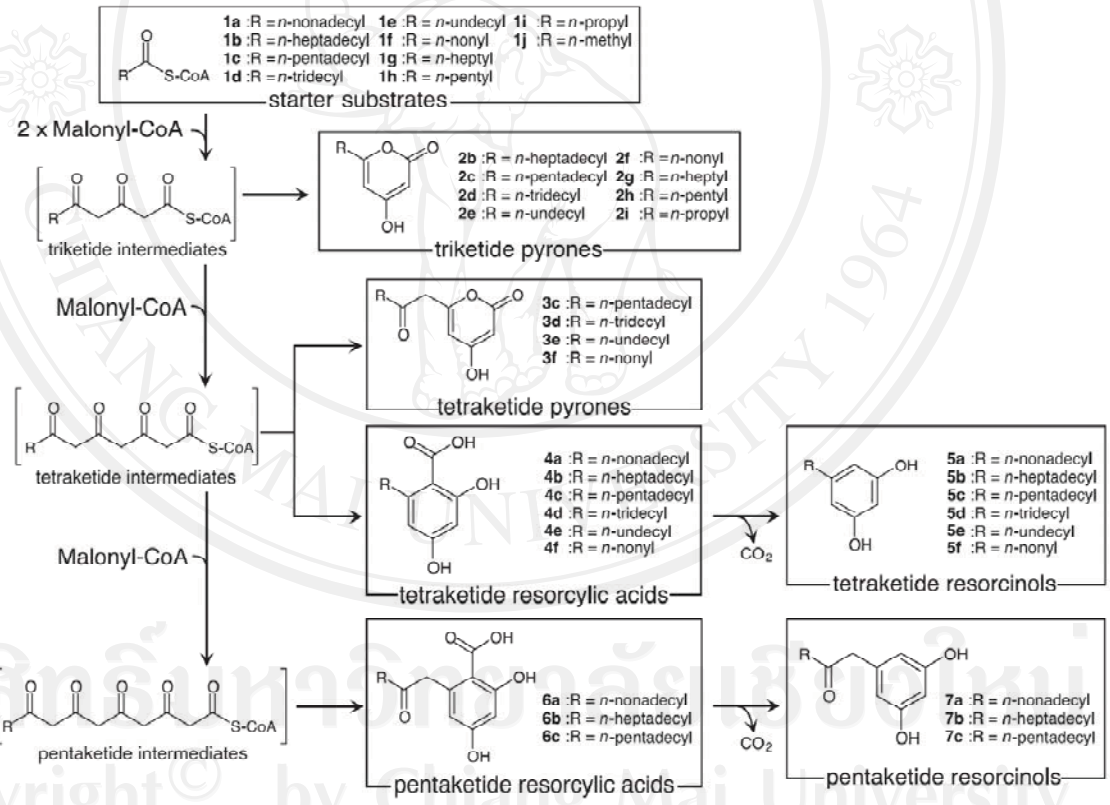

Figure 4.3 Summary of reactions catalyzed by ORAS as a 2′-oxoalkylresorcylic acid synthase (Funa *et al*., 2007)

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Type III PKSs share an evolutionary origin with the type II fatty acid synthase in plants and bacteria. In contrast to the multi-enzyme organization of type II PKSs, type III PKSs use a single KS-like active site to catalyze the repetitive condensation of acetate units to a CoA-derivatized starter molecule, typically yielding mono-and bi-cyclic aromatic products (Austin and Noel, 2003). Chain extension is often followed by intramolecular condensation and aromatization of the linear intermediate, all within the same PKS active site cavity. Type III systems are simple architectures which has enabled rapid development of a mechanistic framework. In this study, no fragments of a KS domain from type III PKSs were amplified, but the results reveal that there are at least two PKSs related to PKS type III. This is the first report that *G. amomi*, has a type III PKS in its genome.

4.4.2 Diversity of fungi related to *pks* gene and potential PK production

Eleven endophytic fungi from Pezizomycotina were identified as potential producers of a wide array of PK compounds. Ketosynthase domain sequences were distributed in reducing PKS subclades II-III. Thus, the phylogram may predict the enzyme system and the possible structural compounds or their derivatives. For example, two KS sequences of *E. shearii* might encode an ortholog of portion PK and it was also found that partial PKS of *E. crustaceum* ZE0151 showed 99% similarity of KS domain isolated from *T. flavus* ZE0199. The endophytic fungus, *Eupenicillium* sp., isolated from the forest tree (*Glochidion ferdinandi*), was investigated and four polyketides, phomoxin, phomoxin B, phomoxin C and eupenoxide, were detected (Davis *et al*., 2005). However, the endophytes *E. shearii* and *E. crustaceum* ZE0151 were isolated from different habitats, i.e. grasses and gingers. In addition, *E. shearii*

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had two homologous KS domain fragments which were classified in a novel clade. Whether these encode enzymes which synthesize the same group of polyketides is uncertain, and needs to be confirmed by metabolite identification. Analysis of metabolites produced by this fungus revealed the presence of two phenolic compounds, phenopyrrozin, and *p*-hydroxyphenopyrrozin.

Eleven KS fragments were found from nine saprobic fungi. These were distributed in all the reducing PKS clades, but mostly found in reducing PKS subclade I with only one fragment being assigned to the non-reducing PKS clade. Two fragments of KS isolated from *Dictyosporium digitatum* were grouped into a novel clade and had a percent identity of amino acid sequences from BLASTp analysis lower than 50% when compared with GenBank database. *Myrothecium pandanicola* obtained two unique KS sequences one of which was surprisingly homologous to non-reducing PKS. This fragment was amplified by highly-reducing PKS primers, and presented a PKS homolog to *Botryotinia fuckeliana* PKS15 which was a member of non-reducing PKS (Kroken *et al.*, 2003). Thus, we need to address that why the amplification had done error or it is such as failure amplification, technically.

Nectria haematococca MSN045 and *Hyponectria manglietiae* MS054 were found to have ortholog partial genes and both were grouped into subclade of lovastatin/citrinin diketide and T-toxin. It was interesting that *Periconia tirupatiensis* showed 98% identity similar to *Monascus purpureus* and these partial PKS were related to fumonisin synthesis which presented in the reducing PKS subclade IV. Finally, we concluded that KS-AT series primer provided the potential to search for PKSs in fungal genomes as a mini tool. KS domain sequences were homologous to those sequences in database (Bingle *et al*., 1999; Kroken *et al*., 2003). Particularly, it

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is interesting that novel strains could produce novel compounds which are synthesized by an enzyme system other than reducing type I PKS. Many tropical fungi probably have a high potential to produce important bioactive compounds especially, the endophytic fungi.

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