Applied and Environmental Microbiology, June 2009, p. 3721–3732 Vol. 75, No. 11
0099-2240/09/$08.00+0 doi:10.1128/AEM.02744-08

Insect-Specific Polyketide Synthases (PKSs), Potential PKS-Nonribosomal Peptide Synthetase Hybrids, and Novel PKS Clades in Tropical Fungi▽†

Alongkorn Amnuaykanjanasin,[1]* Suranat Phonghanpot,[2] Nattapong Sengpanich,[1] Supapon Cheevadhanarak,[2] and Morakot Tanticharoen[1]

National Center for Genetic Engineering and Biotechnology, National Science and Technology Development Agency, 113 Paholyothin Road, Pathumthani 12120, Thailand,[1] and School of Bioresources and Technology, King Mongkut's University of Technology Thonburi, Bangkok 10140, Thailand[2]

Received 2 December 2008/Accepted 28 March 2009

Polyketides draw much attention because of their potential use in pharmaceutical and biotechnological applications. This study identifies an abundant pool of polyketide synthase (PKS) genes from local isolates of tropical fungi found in Thailand in three different ecological niches: insect pathogens, marine inhabitants, and lichen mutualists. We detected 149 PKS genes from 48 fungi using PCR with PKS-specific degenerate primers. We identified and classified 283 additional PKS genes from 13 fungal genomes. Phylogenetic analysis of all these PKS sequences the comprising ketosynthase (KS) conserved region and the KS-acyltransferase interdomain region yielded results very similar to those for phylogenies of the KS domain and suggested a number of remarkable points. (i) Twelve PKS genes amplified from 12 different insect-pathogenic fungi form a tight cluster, although along with two PKS genes extracted from genomes of *Aspergillus niger* and *Aspergillus terreus*, in reducing clade III. Some of these insect-specific fungal PKSs are nearly identical. (ii) We identified 38 new PKS-nonribosomal peptide synthetase hybrid genes in reducing clade II. (iii) Four distinct clades were discovered with more than 75% bootstrap support. We propose to designate the novel clade D1 with 100% bootstrap support "reducing clade V." The newly cloned PKS genes from these tropical fungi should provide useful and diverse genetic resources for future research on the characterization of polyketide compounds synthesized by these enzymes.

One hallmark of tropical countries is the tremendous availability and diversity of natural resources. Tropical forests, freshwater reservoirs, and seas are home to an uncountable number of species, ranging from microorganisms (e.g., bacteria, fungi, and protozoa) to invertebrates to vertebrates to plants. Thailand is no exception. The country has a large collection of fungi found in different niches and habitats in its ecosystems. Interesting groups include fungi that are associated with insects, those that inhabit the sea, and those that are in lichen complexes; these are referred to here as insect fungi, marine fungi, and lichenized fungi, respectively. The first group is of particular interest because it represents a remarkable relationship (in this case, pathogenesis) between the fungi and their insect hosts. These entomopathogenic fungi were isolated from the dead insect bodies in different stages (e.g., larvae, pupae, nymphs, or adults). The marine fungi used in this study were mostly isolated from the living or dead plant parts floating at the seashore, whereas the lichen mutualistic fungi were isolated from lichen complexes on the bark of trees in tropical forests in Thailand. All these fungal isolates were deposited in National Center for Genetic Engineering and Biotechnology (BIOTEC) Culture Collection (BCC). The BCC has one of the richest collections (approximately 400 species and 5,000 isolates) of insect fungi in the world (19).

Secondary metabolites may play an important role in organisms that synthesize them, for example, in spore development (7), protection, or host virulence (5). Polyketides (PKs) are natural secondary metabolite compounds derived from the condensation of acyl coenzyme A subunits in a head-to-tail manner, and they have a tremendous diversity in structure (33). Structural diversification of the PKs includes a variation in the number of subunits, types of subunits, degree of chemical reduction of the β-keto thioester, extent of stereochemistry of the α-keto group at each condensation, and subsequent processing (e.g., cyclization) (25, 28, 33). The high therapeutic and economic value of PK compounds has attracted the interest of drug companies and government research agencies. Some PKs are commercially available for medical treatments, such as grahamimycin and patulin (antibiotics), lovastatin and compactin (cholesterol-lowering agents), griseofulvin (an antibiotic/antifungal agent), and monocerin (an antifungal agent).

Enzymes that synthesize the PKs are called PK synthases (PKSs). PKSs are multifunctional enzymes that are composed of three principal domains: ketoacyl synthase (KS), acyltransferase (AT), and acyl carrier protein (ACP). Fungal PKSs are type I, multifunctional large enzymes and use an iterative strategy to synthesize PKs. They can be divided into two groups, nonreducing (NR) and reducing (4), and further subdivided into NR subclades I, II, and III and reducing subclades I, II, III, and IV (26). NR PKSs include those synthesizing pigments or aflatoxin. Reducing PKSs are involved in the synthesis of PK compounds with various chemical reductions in structure.

* Corresponding author. Mailing address: National Center for Genetic Engineering and Biotechnology, 113 Paholyothin Rd., Pathumthani 12120, Thailand. Phone: 662 5646700. Fax: 662 5646707. E-mail: alongkorn@biotec.or.th.

† Supplemental material for this article may be found at http://aem.asm.org/.

▽ Published ahead of print on 3 April 2009.

Apart from the three major domains (KS, AT, and ACP) present in all PKSs, reducing PKSs contain three additional domains, i.e., dehydratase, enoyl reductase, and ketoreductase, which are involved in the reduction of the keto group to various stages (i.e., alcohol, unsaturated thiolester, and full saturation, respectively), therefore enhancing diversity of the PK structure.

Kroken et al. (26) studied putative amino acid sequences of the PKS genes previously characterized in fungi and the PKS genes discovered from the genome sequencing projects for eight fungal species in the *Ascomycota*. PKS genes were found only in the genomes of the *Pezizomycotina* and not in the sequenced genomes of either *Ascomycota* in the *Taphrinomycota* or *Saccharomycotina* or *Basidiomycota* in the *Hymenomycetes*. Thus, we focused our search on the fungi in this subphylum. We aimed to mine valuable PKS genes from this fungal resource. One of the main objectives is to find novel secondary metabolites useful for medical or agricultural applications. One highly regarded example is the "vegetable caterpillar," where the fungus *Cordyceps sinensis* grows on Hepialidae caterpillars. The fungus has long been used in traditional Chinese medicine. Extracts of *Cordyceps sinensis* were reported to have a variety of therapeutic effects, for example, antitumor (6), antioxidant (42), and antiaging (24) activities. The *C. sinensis*-Hepialidae pair is also called the "body snatcher". This name comes from the fact that the fungus infects and consumes the insect tissue and fills up the insect cavity with its mycelia. Thus, another objective is to find metabolites involved in interaction between fungal pathogens and their insect hosts. Insect pests pose tremendous losses to humans in regard to health issues (vectors of diseases) and economic issues (crop plant losses by insect pathogens and building structure damage by termites). Little was known regarding the roles of PKs in producing fungi on their interaction with insect host. Better understanding of this relationship might have implications for insect control.

We conducted our PKS screening using PCR with the degenerate KA series primers (2). In addition to our preliminary PKS screening with these primers in a few fungi (2), the KA series primers were used to clone the reducing PKS gene for radicicol biosynthesis from the fungus *Pochonia chlamydosporia*, and later its whole biosynthetic cluster was revealed (37). Here, the method and the primers were also proven to be successful in finding rich resources of hidden metabolic pathways for PK biosynthesis from 48 fungi that were isolated in Thailand and, particularly, have no genome sequences determined. In addition, more than 200 PKS genes were identified from our genome analysis of 13 filamentous fungi.

MATERIALS AND METHODS

Fungal strains, culture media, and genomic DNA preparation. Insect-pathogenic, lichenized, and plant-pathogenic fungi were grown at 25°C on potato dextrose agar (Difco) and potato dextrose broth (Difco) for preparation of inoculum and genomic DNA extraction, respectively. Marine-derived fungi were cultured on a modified malt extract agar (Oxoid, United Kingdom) and a modified malt extract broth (Oxoid) in which the powder was solubilized with seawater, which stimulated the growth of the marine fungi.

All the fungal strains used in this study were obtained from the BCC and the Department of Agriculture, Thailand (Table 1). They were maintained by subculturing biweekly on the agar media as described above. *E. coli* strain DH5α was used for maintaining all plasmids. For genomic DNA isolation, fungi were grown in the liquid culture media as described above for 5 to 7 days. The fungal mycelia were lyophilized and ground into powder. Fungal genomic DNAs were extracted according to the method described previously by Reader and Broda (36).

Amplification, cloning, and sequencing of the KS-AT regions. The KS conserved domain and the KS-AT interdomain region (referred to as KS-AT) was amplified from extracted fungal genomic DNA using PCR with the degenerate KA series primers (2). The forward primer KAF1 was paired with the reverse primer KAR1 or KAR2 for PCR amplification of these PKS gene fragments. Approximately 100 ng of fungal genomic DNA was mixed with 1× PCR buffer minus Mg (Invitrogen), 1 unit of recombinant *Taq* DNA polymerase (Invitrogen), 200 μM of each deoxynucleoside triphosphate (Fermentas, Canada), 3.5 mM $MgCl_2$, and 400 nM each of a forward and a reverse primer. Thermal cycling parameter was set for initial denaturation at 95°C for 2 min, followed by 35 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 2 min. Final extension was performed at 72°C for 10 min. Each PCR product was separated by agarose gel electrophoresis, excised, and purified using a QIAquick gel extraction kit or QIAquick PCR purification kit (Qiagen). The purified DNA was ligated into the vector pCR 2.1 (Invitrogen). Further confirmation of the insertion and size of the PCR product insert was conducted using EcoRI excision that released the insert from the cloning vector (Fermentas). Finally, the insert was sequenced by Macrogen (South Korea).

Identification of PKS genes from fungal genomes. We identified all the PKS genes from each of the genomes of 13 ascomycetous fungi: *Aspergillus clavatus* NRRL 1 (14), *Aspergillus fumigatus* 293 (31), *Aspergillus nidulans* FGSC A4 (16), *Aspergillus niger* ATCC 1015, *Aspergillus oryzae* RIB 40 (30), *Aspergillus terreus* NIH 2624, *Chaetomium globosum* CBS 14851, *Magnaporthe grisea* 70-15 (9), *Neosartorya fischeri* NRRL 181 (14), *Phaeosphaeria nodorum* SN 15 (18), *Podospora anserina* (12), *Pyrenophora tritici-repentis*, and *Sclerotinia sclerotiorum* 1980. *A. terreus*, *C. globosum*, *P. tritici-repentis*, and *S. sclerotiorum* were sequenced by the Broad Institute's Fungal Genome Initiative (www.broad.mit.edu), whereas *A. niger* genome sequencing was conducted at the DOE Joint Genome Institute (genome.jgi-psf.org/Aspni5). These fungi include three human pathogens, four plant pathogens, two model fungi, and four industrial fungi for protein/metabolite production. We performed annotated and BLAST (Basic Local Alignment Search Tool, National Center for Biotechnology Information [NCBI]) searches against each fungal genome. For the annotated search, PKS genes were retrieved using keywords "polyketide synthase" and fungal species names in Entrez, Protein and Gene (NCBI). For the BLAST search, the KS-AT region of a known PKS, AtLovF (without the KA series primers) was used as query in tBlastN or BlastP of genomic BLAST (NCBI). The hits with E values of less than 1e−10 were considered to have significant homology to PKS genes. The KS-AT regions were extracted from deduced amino acid sequences of these PKS genes. Putative proteins of PKS genes that have fewer than 1,000 amino acids in their total lengths and do not contain ExHGTGT, EAHATST, or closely related sequences and FSGHGAQW, FTGQGAQW, or closely related sequences (the conserved regions for the forward and reverse KA series primers, respectively) were not included in this study.

Sequence and phylogenetic analysis of PKS fragments. Sequences of the cloned fragments were searched for similarity to known proteins using BLAST search (NCBI). Putative introns were predicted using similarity search results in BLASTX and the presence of the GT/AG 5′ and 3′ intron splicing sites (21). The software Genetyx (www.sdc.co.jp/genetyx) was used to digitally excise putative introns. All amplified PKS sequences were deposited in NCBI's GenBank databases. Reference amino acid sequences of each reducing/NR class of PKSs published in the literature were retrieved from NCBI database and used in the phylogenetic analysis (Fig. 1). Their accession numbers are given in Table S2 in the supplemental material. All the amplified PKS fragments without the KA series primers (except AkpF1R2A1, which is too divergent from the rest) and the retrieved reference PKS sequences were aligned using CLUSTALX package 1.81 (41). In addition, another multiple-sequence alignment was performed using the same set of PKS sequences described above as well as all the PKS genes retrieved from the 13 fungal genomes. The sequence alignments from CLUSTALX were edited using BIOEDIT version 7.0.4.1 (17) and exported in NEXUS format, in order to perform phylogenetic analysis using PAUP version 4.0b10 (40).

To perform neighbor-joining (NJ) analysis, the alignment file can be directly analyzed using the NJ method with Kimura two-parameter model. For maximum-parsimony (MP) analysis, the alignment was used to search for the best tree via heuristic search, random stepwise addition of 10 replicates, and the tree-bisection-reconnection branch-swapping algorithm. Gaps were treated as missing data, and the Kishino-Hasegawa test was used to analyze the most parsimonious tree. After the trees were obtained from both NJ and MP analyses, the phylogram with branch length was saved in Newick format and viewed with TreeViewX version 1.6.6 (darwin.zoology.gla.ac.uk/~rpage/treeviewx).

TABLE 1. All 48 fungi used in this study[a]

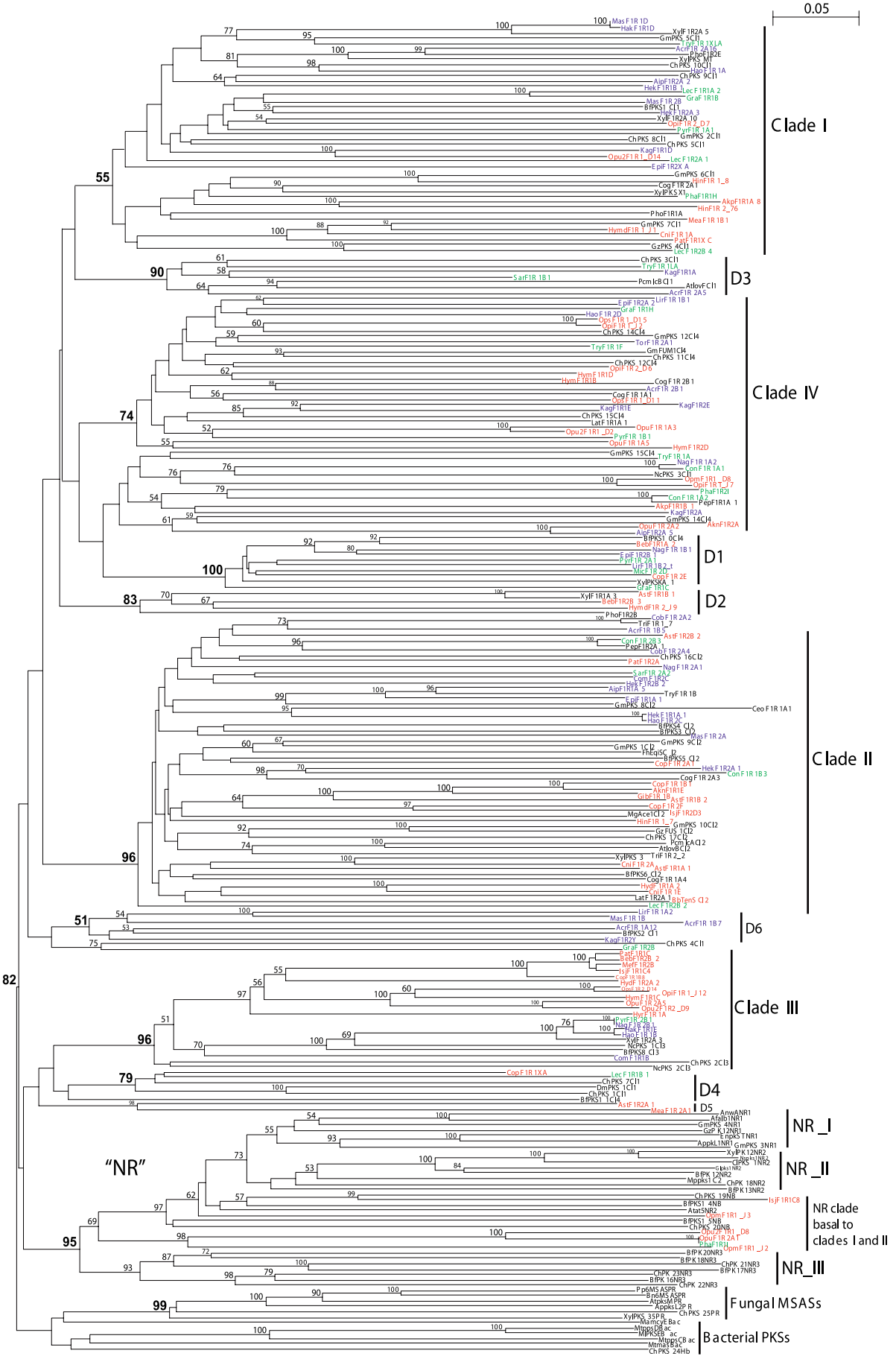
Fungus	Code[b]	Taxonomic class	Habitat type[c]	Substrate where found
Akanthomyces novoguineensis BCC2837	Akn	Sordariomycetes	Insect	Araneae (spider)
Akanthomyces pistillariformis BCC1428	Akp	Sordariomycetes	Insect	Lepidoptera (adult moth)
Aschersonia tubulata BCC7681	Ast	Sordariomycetes	Insect	Homoptera (nymph)
Beauveria bassina BCC1625	Beb	Sordariomycetes	Insect	Insecta (pupa)
Cordyceps nipponica BCC1389	Cni	Sordariomycetes	Insect	Neuroptera (nymph)
Cordyceps pseudomilitaris BCC1620	Cop	Sordariomycetes	Insect	Lepidoptera (larva)
Gibellula sp. BCC14132	Gib	Sordariomycetes	Insect	Araneae (spider)
Hirsutella nivea BCC2594	Hin	Sordariomycetes	Insect	Homoptera (hopper)
Hymenostilbe sp. BCC1571	Hym	Sordariomycetes	Insect	Isoptera (termite)
Hymenostilbe dipterigina MY1105	Hymd	Sordariomycetes	Insect	Diptera (fly)
Hypocrella discoidea BCC8239	Hyd	Sordariomycetes	Insect	Homoptera (scale insect)
Hypocrella raciborski BCC17141	Hyr	Sordariomycetes	Insect	Homoptera (scale insect)
Isaria javanica BCC29250	Isj	Sordariomycetes	Insect	Lepidoptera (larva)
Metarhizium anisopliae BCC1617	Mea	Sordariomycetes	Insect	Homoptera (hopper nymph)
Metarhizium flavoviride BCC7623	Mef	Sordariomycetes	Insect	Homoptera (hopper nymph)
Ophiocordyceps irangiensis BCC21423	Opi	Sordariomycetes	Insect	Hymenoptera (ant)
Ophiocordyceps myrmecopphila MY163	Opm	Sordariomycetes	Insect	Hymenoptera (ant)
Ophiocordyceps sphecocephala BCC21472	Ops	Sordariomycetes	Insect	Hymenoptera (ant)
Ophiocordyceps unilaterlais BCC14750	Opu	Sordariomycetes	Insect	Hymenoptera (ant)
Ophiocordyceps unilateralis KT3307	Opu2	Sordariomycetes	Insect	Hymenoptera (ant)
Paecillomyces tenuipes BCC1614	Pat	Sordariomycetes	Insect	Lepidoptera (pupa)
Acremonium sp. strain BCC9257	Acr	Sordariomycetes	Marine	Wood
Aigialus parvus BCC6195	Aip	Dothideomycetes	Marine	Mangrove
Corollospora besarispora BCC6732	Cob	Sordariomycetes	Marine	
Corollospora maritima BCC12026	Com	Sordariomycetes	Marine	Sand grain
Epicoccum sp. strain BCC8702	Epi	Dothideomycetes	Marine	Mangrove
Halorosellinia oceanica BCC11430	Hao	Sordariomycetes	Marine	Wood
Halosarpheia cf. kandeliae BCC13120	Hak	Sordariomycetes	Marine	Wood
Helicascus kanaloanus BCC13107	Hek	Dothideomycetes	Marine	*Sonneratia*
Kallichroma glabrum BCC13115	Kag	Sordariomycetes	Marine	Mangrove
Lineolata rhizosphorae BCC11179	Lir	Dothideomycetes	Marine	Driftwood
Massarina sp. strain BCC18015	Mas	Dothideomycetes	Marine	Submerged coconut
Nais glitra BCC11151	Nag	Sordariomycetes	Marine	Mangrove
Torpedospora radiata BCC11269	Tor	Ascomycota incertae sedis	Marine	Driftwood
Coniothyrium sp. strain BCC3073	Con	Sordariomycetes	Lichen	Lichen
Graphis afzelii BCC12103	Gra	Lecanoromycetes	Lichen	Bark
Lecanora sp. strain BCC12113	Lec	Lecanoromycetes	Lichen	Bark
Microsphaeropsis sp. strain BCC3050	Mic	Anamorphic Ascomycota	Lichen	*Dirinaria applanata* lichen
Phaeographina sp. strain BCC12100	Pha	Lecanoromycetes	Lichen	Bark
Pyrenula sp. strain BCC12115	Pyr	Eurotiomycetes	Lichen	Bark
Sarcographa sp. strain BCC12132	Sar	Lecanoromycetes	Lichen	Bark
Trypethelium sp. strain BCC9790	Try	Eurotiomycetes	Lichen	Bark
Cercospora oryzae	Ceo	Dothideomycetes	Plant	Rice
Colletotrichum gleosporiodes BCC13327	Cog	Sordariomycetes	Plant	Banana
Lasiodiplodia theobromae	Lat	Dothideomycetes	Plant	Cocoa
Pestalotiopsis psidii	Pep	Sordariomycetes	Plant	Guava
Phomopsis sp. strain BCC9451	Pho	Sordariomycetes	Endophyte	*Lagerstroemia* leaves
Trichoderma sp. strain BCC7579	Tri	Sordariomycetes	Soil	Soil

[a] Data are from Thailand's BIOTEC Culture Collection and Department of Agriculture.
[b] Abbreviation used in designations in the text.
[c] Insect, insect pathogen; marine, marine inhabitant; lichen, lichenized fungus; plant, plant pathogen; endophyte, plant endophyte; soil, soil inhabitant.

Nucleotide sequence accession numbers. Cloned PKSs with their GenBank accession numbers are provided in Table S1 in the supplemental material.

RESULTS AND DISCUSSION

One hundred forty-nine PKS genes in 48 tropical fungi. We screened a total of 48 filamentous ascomycetes that live in different habitats and include pathogens of insects, inhabitants of the ocean, symbionts with blue-green algae in the lichen complex, and phytoendophytic (a *Phomopsis* sp.) or phytoparasitic (*Cercospora oryzae* or *Colletotrichum gleosporiodes*) fungi (Table 1). All of these 48 fungal isolates contained at least one PKS gene fragment according to the PCR amplification with KA series primers. In this study, we found 149 PKS gene fragments from this group of fungi, an average of 3.1 PKSs detected per fungus. Nearly all (96%) of these fragments belonged to the reducing PKSs, the expected targets of the KA series primers. Newly cloned PKSs were distributed into the four reducing clades identified earlier (24): clades I (20% of the total number of reducing PKSs), II (25%), III (11%), and IV (27%) (Table 2). Fourteen percent of these reducing PKSs



were classified into new clusters (D1, D2, D3, D4, D5, and D6 in Fig. 1 and Table 2) according to our phylogeny. However, six PKS gene fragments (4% of the total number of all PKS genes) are within the main NR clade, including IsjC8, OpmF1R1_J2, OpmF1R1_J3, OpuF1R2A1, Opu2F1R1_D8, and PhaF1R1l. All these NR PKS fragments are from insect-pathogenic fungi. They appeared to be placed with the NR clade in both NJ and MP analyses (the NJ tree is shown in Fig. 1). These PKS fragments from *Isaria javanica*, *Phaeographina* sp., and the genus *Ophiocordyceps* were in the NR clade with 95% bootstrap support (Fig. 1).

The PKS fragments identified by the KA series primers have a nucleotide length of 0.5 to 1.2 kb (Fig. 2 and Table 2). The most common sizes of these fragments are between 0.61 and 0.8 kb (54% of all the cloned fragments) with either no, one, or two introns (Table 2). Other sizes include 0.51 to 0.60 kb (8%), 0.81 to 1.00 kb (35%), and 1.01 to 1.02 kb (3%). All the larger fragments (>0.80 kb) contained one to four introns; many of the introns in these fragments were more than 100 bp (Table 2). Thus, the deduced amino acid sequences of these PKS fragments were usually 200 to 300 amino acids in length after removal of putative intron(s). Other studies tended to discard the PCR fragments larger than their expected sizes without sequencing to confirm whether these were actual PKS genes (>0.7 to 0.8 kb [4] and >0.3 kb [27]). Here, we report that most of the large fragments were also PKS genes, and the larger size could just be due to the presence of an intron(s) in those fragments. In addition, among the 48 fungi we screened, the highest number of PKS genes found in one single fungus was six. There were three fungi that contained six PKSs according to PCR with KA series primers (*Acremonium* sp. strain BCC9275, *Cordyceps pseudomilitaris*, and *Kallichroma glabrum*); two of these are marine fungi.

The forward primer KAF1 used in this study was derived from the same conserved region (ExHGTGT) in the KS domain as that of LC2c or LC5c (4). However, our PCR amplification result indicated that the KAF1 primer paired with KAR1/2 primers (from the AT domain) detected considerably more PKS genes per fungus than LC2c or LC5c paired with LC1 or LC3 (4, 27, 29). All the LC primers were generated only from the KS domain. The difference in number of obtained PCR products could be due to differences in PCR conditions, including magnesium concentration, type of *Taq* DNA polymerase, thermal cycling parameters, or amplification efficiency and compatibility of pairs of forward and reverse primers.

The KA series primers appeared to detect fewer reducing PKS genes (1 to 6 [Table 2]) than those found in our analysis of the sequenced fungal genomes (9 to 28, [Table 3]). This result was expected and was accounted for in our analysis of fungal genomes by the finding that the conserved regions $EA_{(c)}$HGTGT and FTGQGAQW/FSGHGAQW for the primers KAF1 and KAR1/2, respectively, are not present in many PKS genes in these genomes. Nonetheless, the average number of three PKS genes detected per fungus should suggest that PCR with KA series primers can be one efficient method to identify PKS genes from fungi whose genome sequences are not available. Two isolates of *Ophiocordyceps unilateralis* were included in our PKS screening, and both have four PKS genes detected with the primers (Table 2). Two of the four genes in each of the *O. unilateralis* isolates appeared to be homologous: OpuF1R2A5-Opu2F1R2D9, in reducing clade III, and OpuF1R1A3-Opu2F1R1_D2, in clade IV. Furthermore, we detected a PKS homolog in each of three isolates of *Beauveria bassiana* screened, and they were also in the reducing clade III (data not shown). Although our PCR conditions may not be optimal for all PKS genes, the KA series primers still could find a homologous gene probably common in different isolates of the same fungal species.

PKS genes in the genomes of filamentous fungi. To determine the presence and distribution of PKS genes in other fungi for which fungal genome sequences are available, we extracted 283 PKS genes from the 13 fungal genomes (Table 3). Accession numbers of these PKSs are provided in Table S3 in the supplemental material. It is noted that we discarded the genes that appeared to be PKS-like (not full PKS proteins and not containing a full set of PKS minimal domains [KS-AT-ACP]). The number of PKS genes in each fungal genome was reported previously (8). Here, we elaborated the classification of these genomic PKSs into subgroups. The majority (71%) of these PKSs are of the reducing type. They were classified into the four reducing clades (24): clades I (22% of the total number of reducing PKSs), II (23%), III (5%), and IV (24%) (Table 3; see Fig. S1 in the supplemental material). The distribution of these genomic PKSs in the four reducing clades was fairly similar to that of our newly cloned PKSs (Table 2). Twenty-five percent of these reducing PKSs were classified into the novel clades according to our phylogeny. *Aspergillus oryzae* and *A. terreus* have at least one PKS in each of reducing clades I to IV and distinct clades D1 to D4. Approximately one-fourth of the PKS genes from these fungal genomes are within the main NR clade. *A. niger* has 94% of reducing PKSs, compared with the average of 74% in this group of fungi. The fungus has a very high number of PKSs in each of the reducing clades I, II, and IV (Table 3). *A. niger* is an industrial fungus used as a source for production of citric acid and many hydrolytic and oxidative enzymes. Also, the fungus is very commonly found in the environment (3). This may account, in part, for a wide array of PKS enzymes possibly for biosynthesis of various types of PKs for various ecological purposes. *A. clavatus* also has multiple PKSs in each of clades I and II.

Our KS-AT phylogeny is similar to the KS phylogenies and gives reliable predictions of PKS functions. Deduced amino acid sequences from the C terminus of the KS domain and the KS-AT interdomain region of the cloned PKS genes were used to generate multiple alignments and phylogenetic trees. These

FIG. 1. NJ phylogenetic tree of deduced amino acid sequences of 149 fungal PKS gene fragments (amplified with KA series primers; marked in red, blue, and green for insect, marine, and lichenized fungi, respectively) and other known NR and reducing fungal PKSs and bacterial PKSs in the NCBI database (in black). Bacterial PKSs were used as the outgroup in the tree. Bootstrap analysis using NJ was conducted with 1,000 replicates, and values of ≥50% are given at nodes.

TABLE 2. PKS genes cloned from 48 fungi using PCR amplification with KA series primers

Habitat type and fungus code[a]	No. of PKS genes[b]	Sizes of PKS genes (kb)	Intron sizes (bp)[c]	No. of genes in reducing clade:					No. of genes in NR clade
				I	II	III	IV	Novel[d]	
Insect									
Akn	2	0.70, 0.82	no, (70, 60)		1		1		
Akp	2	0.78, 0.70	no, no	1			1		
Ast	5	0.80, 0.58, 0.73, 0.99, 0.71	no, 59, no, (55, 68, 61), no		3			D2, D5	
Beb	3	0.89, 0.71, 0.70	(47, 52, 74), no, no			1		D1, D2	
Cni	3	0.82, 0.75, 0.76	50, no, no	1	2				
Cop	6	0.72, 0.90, 0.71, 0.77, 0.71, 0.87	no, no, no, no, no, 55		3	1		D1, D4	
Gib	1	0.74	30		1				
Hin	3	0.75, 0.83, 0.80	58, 40, no	2	1				
Hym	4	0.66, 0.75, 0.69, 0.80	no, 40, no, (60, 60)			1	3		
Hymd	2	0.52, 0.54	no, no	1				D2	
Hyd	2	0.77, 0.69	no, no		1	1			
Hyr	1	0.70	no			1			
Isj	3	0.70, 0.56, 0.80	no, no, 87		1	1			1
Mea	2	0.81, 0.71	no, no					D4, D5	
Mef	1	0.71	no			1			
Opi	5	0.7, 0.82, 0.85, 0.82, 0.73	(56, 61), 168, no, (85, 60), no	1		1	3		
Opm	3	0.83, 0.73, 0.7	(56, 60), no, no				1		2
Ops	3	0.82, 0.70, 0.83	(49, 53, 51), no, 131			1	2		
Opu	4	0.95, 0.85, 0.71, 0.76	(48, 50, 68), (53, 58, 61), 65, (47, 53)			1	3		
Opu2	4	0.73, 0.79, 0.81, 0.7	(50, 55, 57), no, 83, no	1		1	1		1
Pat	3	0.70, 0.95, 0.76	no, (62, 69), 40	1	1	1			
Marine									
Acr	6	1.02, 0.80, 0.76, 1.02, 0.97, 0.73	104, 62, no, 262, 54, 37	1	1		1	D3, D6(2)	
Aip	3	0.77, 0.84, 0.79	60, no, 100	1	1		1		
Cob	2	0.80, 0.72	60, no		2				
Com	2	0.70, 0.87	no, (80, 50)		1	1			
Epi	4	0.76, 0.67, 0.95, 0.89	no, no, 120, 51	1	1		1	D1	
Hao	4	0.82, 0.76, 0.81, 0.67	62, 48, 53, no	1	1	1	1		
Hak	2	0.90, 0.75	no, 40	1		1			
Hek	4	0.73, 0.83, 0.94, 0.72	no, 63, (49, 53), no	2	2				
Kag	6	0.85, 0.66, 1.01, 0.73, 0.70, 0.66	no, no, 50, no, no, no	1			3	D3, D6	
Lir	3	0.81, 0.65, 0.88	83, no, (52, 48)				1	D1, D6	
Mas	4	0.79, 0.79, 0.75, 0.77	no, no, no, no	2	1			D6	
Nag	4	0.95, 0.84, 0.93, 0.71	(62, 57), (57, 67), 169, no		1	1	1	D1	
Tor	1	0.76	31				1		
Lichen									
Con	4	0.95, 0.89, 0.78, 0.81	(62, 57), 141, no, no		2		2		
Gra	4	0.92, 1.15, 0.71, 0.96	no, (200, 50, 100), no, no	1			2	D1	
Lec	5	1.01, 0.75, 0.88, 0.71, 0.71	(94, 48, 56), no, 49, no, no	4	1				
Mic	1	0.88	110					D1	
Pha	3	0.79, 0.72, 0.82	no, no, (50, 45)	1			1		1
Pyr	3	0.93, 0.66, 0.93	(50, 54), no, (58, 97, 53)	1			1	D1	
Sar	2	0.51, 0.78	no, 46		1			D3	
Try	5	1.00, 0.75, 0.69, 0.74, 0.79	100, 70, no, no, no	1	1		2	D3	
Plant									
Ceo	1	0.89	(105, 55)		1				
Cog	5	0.89, 0.75, 1.06, 0.80, 0.70	(67, 51, 56, 50), no, (50, 258), no, no	1	2		2		
Lat	2	0.81, 0.73	(56, 47, 53), no		1		1		
Pep	2	0.81, 0.81	(41, 69), no		1		1		
Other									
Pho	3	0.79, 0.74, 0.9	50, no, 162	2				D2	
Tri	2	0.80, 0.87	60, 106		2				

[a] Fungus codes are given in Table 1.
[b] PCR products were determined by similarity searches using BLASTX, and the number of PKS genes detected in each fungus is indicated.
[c] Putative intron sizes correspond to the PKS gene fragments in the adjacent column. "no" indicates that the gene fragment had no introns. Intron sizes in parentheses include the sizes of all the introns found in one PKS gene fragment.
[d] PKS genes belonging to novel reducing clades D1, D2, D3, D4, D5, and D6. If more than one PKS gene is present in each novel clade, the number of PKS genes is in parentheses.

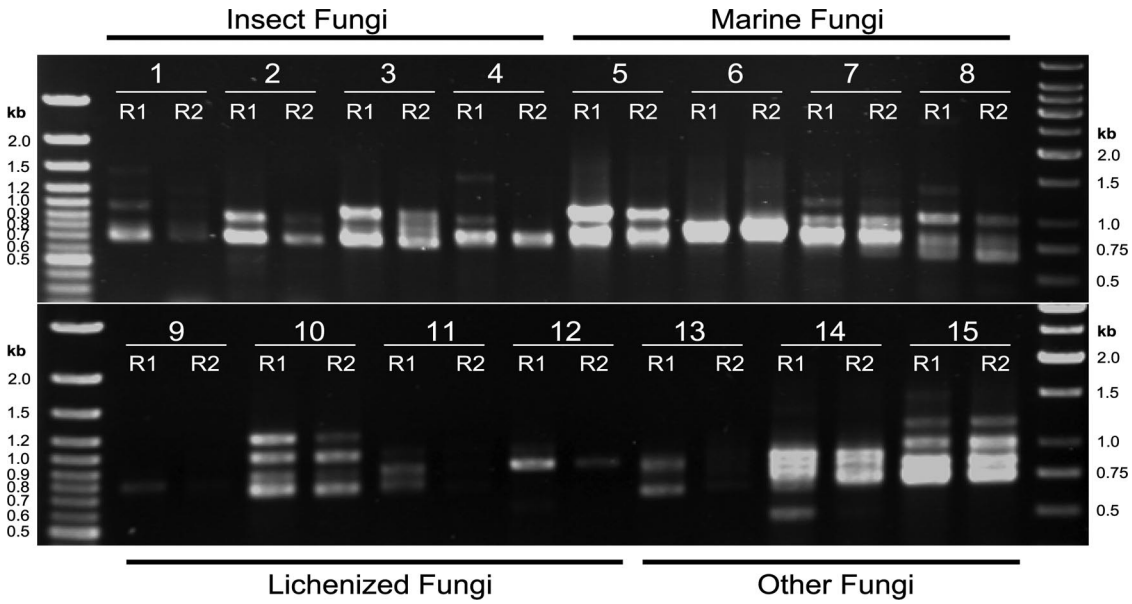


FIG. 2. PCR amplification of PKS genes. Genomic DNA isolated from *Pezizomycotina* fungi was amplified using the KA series primers KAF1-KAR1 (R1) and KAF1-KAR2 (R2). Duplicate lanes (R1 and R2) are shown for each fungus. Insect-pathogenic fungi: 1, *A. pistillaforimis*; 2, *B. bassiana*; 3, *C. pseudomilitaris*; and 4, *P. tenuipes*. Marine fungi: 5, *Acremonium* sp.; 6, *Aigialus parvus*; 7, *H. oceanica*; and 8, *K. glabrum*. Lichenized fungi: 9, *Coniothyrium* sp.; 10, *G. afzelii*; 11, *Microsphaeropsis* sp.; and 12, *Lecanora* sp. Other fungi: 13, *C. gleosporiodes*; 14, *Phomopsis* sp.; and 15, *Xylaria* sp.

PKS fragments contain approximately 110 to 120 amino acids of the KS domain and 100 to 110 amino acids of the KS-AT interdomain region. Mayer et al. (29) commented that there might be a limit to using our KA series primers in PKS gene searches and in classification of the resulting PKS fragments, perhaps due to their interpretation of the KS-AT region that we used. Despite its higher sequence variation than the KS domain, the KS-AT interdomain regions also indicated a pattern and gaps belonging to each PKS clade (those of reducing clade III are shown in Fig. S2 in the supplemental material). Most residues in the analyzed KS-AT regions were parsimony informative with the PAUP software (251 informative versus 4 variable/uninformative and 6 constant characters). Furthermore, our tree generated by the KS-AT region matched the ones produced by the phylogenetic analyses of the KS domain alone (26, 27, 29). Most of the PKS genes or gene fragments classified and designated in the study by Kroken et al. (26) still fall into the same reducing and NR clades in our trees. Regardless of the method we used (NJ or MP), the groupings of nearly all PKSs were highly similar. Clustering of the same groups of PKSs in reducing clades as well as NR clades was usually observed.

TABLE 3. PKS genes in 13 fungal genomes

Fungus	No. of PKS genes[a]																
	Total	NR clades					Reducing clades										Bacterial PKS[d]
		Subtotal	I	II	III	IV[b]	Subtotal	I	II	III	IV	D1	D2	D3	D4	Other[c]	
Aspergillus clavatus	21	4	1		2	1	17	4	7		2			2	1	M	
Aspergillus fumigatus	15	6	1	1	2	2	9	1	3		3	1			1		
Aspergillus nidulans	27	13	3	1	5	4	14	3	2		4	1		2	1	D6	
Aspergillus niger	30	2	1			1	28	10	6	2	8		2				
Aspergillus oryzae	22	6	3		1	2	16	2	4	1	5	1	1	1	1		
Aspergillus terreus	30	10	2	2	4	2	20	3	2	1	5	1	2	3	1	D5, M	
Chaetomium globosum	26	7		1	5	1	18	1	6	2	3			3	2	D5	1
Magnaporthe grisea	21	4		1	1	2	16	7	4	2	2	1					1
Neosartorya fischeri	17	7	1	2	2	2	10	1	2		4	2			1		
Phaeosphaeria nodorum	23	7	2	1	2	2	16	2	2	1	7	1		1		D5, M	
Podospora anserina	20	6	1	1	3	1	14	1	4	1	4		1	1		D5(2)	
Pyrenophora tritici-repentis	15	4		2	1	1	11	5	3					1		D6, M	
Sclerotinia sclerotiorum	16	3	1	2			13	4	2	1	2	1				D6(3)	

[a] PKS genes were determined using annotated and BLAST searches (NCBI) against each fungal genome.
[b] The NR clade basal to NR clades I and II in the phylogenetic tree (Fig. 1).
[c] PKS genes belonging to other reducing clades: D5, D6, and MSAS group (M). If more than one PKS gene is present in each of these clades, the number of PKS genes is in parentheses.
[d] The PKS gene is clustered with the bacterial PKS group.

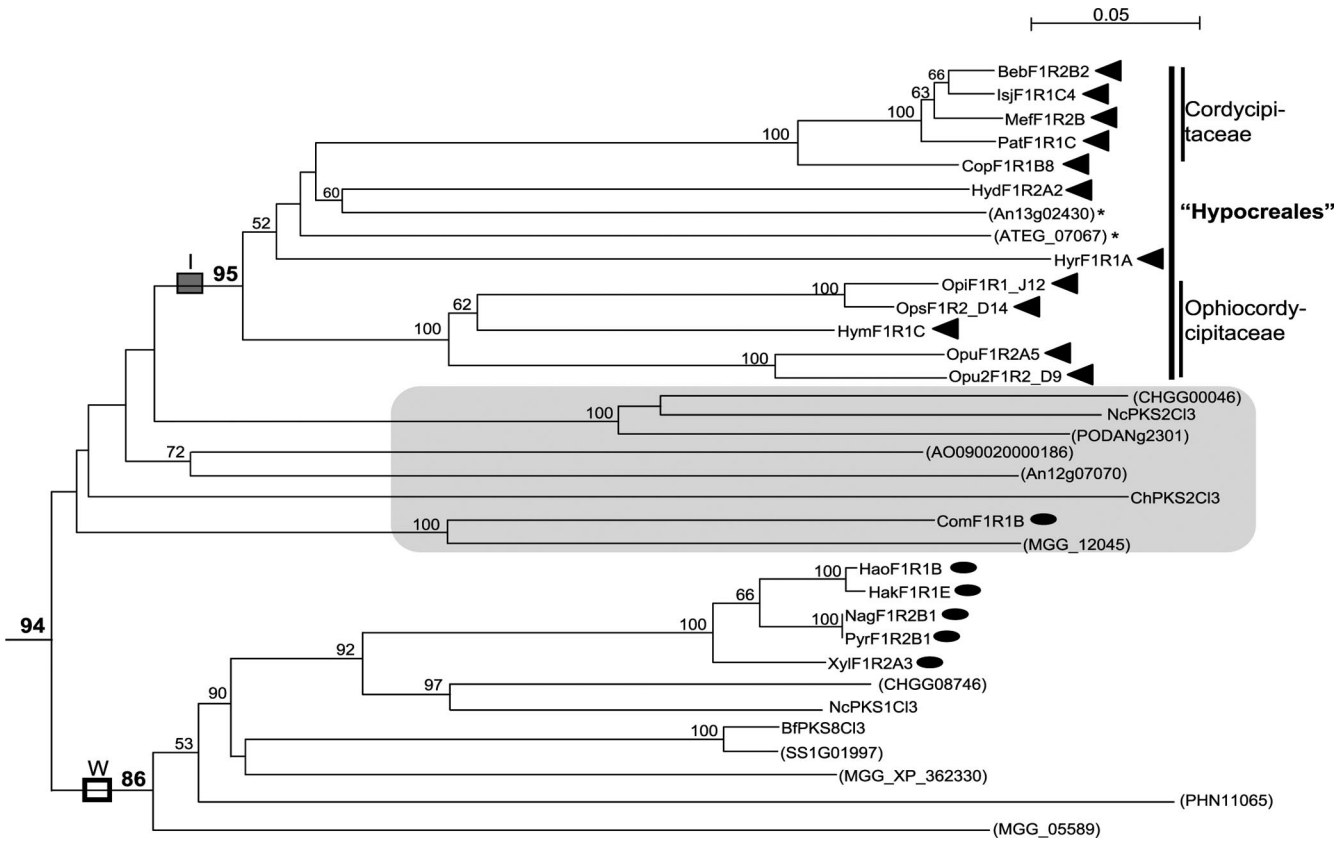


FIG. 3. NJ tree of reducing clade III PKSs. The insect-specific clade III group I and the wood/plant-specific group W are shown on the tree. The KA primer-amplified PKS fragments in groups I and W are indicated by triangles and by black ovals, respectively. PKSs from fungal genomes are in parentheses. All PKSs in group I are from the fungi in the order *Hypocreales*, except for two *Aspergillus* PKSs from the order *Eurotiales* (marked with asterisks). The subgroup basal to the insect-specific group is indicated in the shaded area; these PKSs are mostly genomic. Bootstrap values of ≥50% are given at the nodes from the analysis using NJ with 1,000 replicates.

In many cases, our phylogenetic trees could correctly and strongly group PKSs responsible for biosynthesis of the same type of compounds. For example, PKSs for compactin and lovastatin (both inhibiting 3-hydroxy-3-methylglutaryl-coenzyme A reductase and used as cholesterol-lowering agents), AtLovB and PcMlcA (nonaketide synthases), and AtLovF and PcMlcB (diketide synthases) were tightly clustered (100% bootstrap support) (Fig. 1). GzFUS1 has sequence similarity to a PKS from another *Fusarium* sp., GmPKS10, that synthesizes fusarin C. In our trees, GzFUS1 and GmPKS10 were grouped closely together (Fig. 1), with 78% identity between them and 100% bootstrap support. In a recent study by Gaffoor et al. (15), the functional analysis of Gzfus1 confirmed that the enzyme GzFUS1 was involved in the biosynthesis of the same compound. In our phylogenetic tree, the PKS fragment IsjF1R1C8 is clustered with PTRGpig3 from *Pyrenophora trintici*, which is involved in biosynthesis of a yellow pigment. Interestingly, the fungus *Isaria javanica* also produces the yellow color in culture (its main morphological characteristic) (data not shown).

Insect- and wood-specific reducing clade III PKSs. Although clade III was found in our tested fungi with the lowest frequency among four major reducing clades, it is the cluster with the highest sequence similarity among its members. Reducing clade III was also the only group where relationships between PKSs and fungal habitat could be strongly drawn. This clade III can be classified to the insect-specific group, the group basal to the insect-specific group, and the wood/plant-specific group (Fig. 3). The insect-specific subclade of clade III included 12 members from 12 entomopathogenic fungi (Fig. 1 and 3). They were always clustered with 97% bootstrap support (Fig. 1). PKS gene fragments in this subgroup are so similar that their branch lengths in the phylogenetic tree (Fig. 1) were even shorter than the genetic distance between pairs of PKS orthologs, i.e., compactin and lovastatin synthases (AtLovB versus PcMlcA [35% distant] and AtLovF and PcMlcB [47%]). Four PKSs in this group from *B. bassiana*, *I. javanica*, *M. flavoviride*, and *P. tenuipes* are extremely similar (less than 4% distant). Isolates of *B. bassina* and *M. flavoviride* have been widely used in biological control of insect pests (1, 34). However, to further determine whether this subclade is really insect specific, we included all 283 PKS genes that we found from 13 genomes of fungi in various habitats in a comparable phylogenetic analysis (the whole phylogeny not shown here; see Fig. S1 in the supplemental material). The result indicated that there are only two PKSs from two different fungi falling into this insect-specific group (Fig. 3). These are An13g02430 and ATEG_07067 from the widely used metabolite producers *A. niger* and *A. terreus*, respectively. None of the screened plant

TABLE 4. Conserved amino acid residues of reducing clade III PKSs specific for different groups of fungi

Domain	Relative positions[a]	Conserved domain in the alignment[b]	Conserved residues			
			All insect-pathogenic fungi	Insect biocontrol fungi[c]	*Ophiocordyceps* genus	Wood-inhabiting fungi
KS	65/65	a	$E_{(O)}KA$			$KH_{(T)}P$
	74/74	b	$LDE_{(D)}$			$FKE_{(A)}$
	116/116	c		LS		FT
Interdomain	132/129/132	d		$V_{(I)}VPR$	$-HK_{(R)}$	PTRK
	139/139	e		SGNDEGAAKRNATAI		TANDKASLEAVLKNL
	159/159	f	H			R
	167/167	g		RIIR		$ALMA_{(S)}$
	178/178	h	$ERRT_{(S)}H$			QRRSL
	183/179/183	i		$HS_{(N)}Y$	$L_{(M)}PW$	LQW
	199/199	j		STLNSEAM		$EAINGQR_{(K)}L_{(P)}$

[a] Relative position using the residue number of the first member of group in the multiple alignment: IsjF1R1C4 (all insect-pathogenic fungi and insect biocontrol fungi), Opu2F1R2_D9 (*Ophiocordyceps* genus), and PyrF1R2B1 (wood-inhabiting fungi).
[b] Conserved domains are shown in the multiple-sequence alignment in Fig. S2 in the supplemental material.
[c] The insect biocontrol fungal group includes *B. bassina*, *C. pseudomilitaris*, *I. javanica*, *M. flavoviride*, and *P. tenuipes*.

pathogen and human pathogen genomes contained the insect-specific clade III PKS genes.

The wood/plant-specific subgroup in clade III has five PKSs from amplification with KA series primers (Fig. 3). These fungal sources are apparently from wood/plant-inhabiting origin (three wood-colonizing marine fungi, one lichenized fungus, and one wood-decaying fungus). The five PKSs show very high similarity (92 to 98% identity) in 222 amino acid sequences. Furthermore, seven other PKSs from fungal genomes were placed within this wood/plant-specific clade III groups (Fig. 3) and also plant related (e.g., four major plant pathogens, *B. fuckeliana*, *M. grisea*, *P. nodorum*, and *S. sclerotiorum*, and one common soil inhabitant with ability to decompose plant material, *C. globosum*), except *N. crassa* PKS1.

When we performed phylogenetic analysis of both the newly cloned PKSs and the genomic PKSs, we found that seven genomic PKSs from *A. niger*, *A. oryzae*, *C. globosum*, *C. heterostrophus*, *M. grisea*, *N. crassa*, and *P. anserina* as well as one newly cloned PKS, ComF1R1B, were loosely clustered (with significantly less than 50% bootstrap support) in the basal clade to the insect-specific clade III (Fig. 3). This indicated that although these PKSs were placed in clade III with strong bootstrap support (94% in Fig. 3), they do not belong to either the I or W group.

The very conserved sequences of reducing clade III PKSs leads to the extremely tight cluster of PKSs from insect-pathogenic fungi, particularly the group from *B. bassiana*, *C. pseudomilitaris*, *I. javanica*, *M. flavoviride*, and *P. tenuipes*, compared with other clades. The reason for this remarkable insect-fungal PKS relationship could be because of a specific need of these fungi to preserve the clade III PKS sequence for their ecological needs. Our hypothesis is that the reducing clade III PKSs help fungi infect their insect host; the unique natural metabolite produced by these enzymes is, perhaps, essential for insect pathogenesis. This scientific question is challenging and is being investigated by our research group. Although analysis of fungal genomes suggested the presence of two PKSs from apparently non-insect-pathogenic fungi in the I group (Fig. 2), it may suggest that the two *Aspergillus* species may be opportunistic/rare insect pathogens. Although *A. niger* is less known as being insect pathogenic, metabolites of *A. niger* IHCS-4 had insecticidal activity against *Chrysomya chloropyga* larvae (13) and termites (39). In contrast, no literature showed any insecticidal activity or insect colonization by *A. terreus*.

It is well known that in a PKS the KS domain is the most conserved. In contrast, the KS-AT interdomain region is usually more variable. However, the interdomain parts of the reducing clade III PKSs still remained highly conserved (see Fig. S2 in the supplemental material). The alignment within this region identifies conserved regions (Table 4) specific for all-insect fungal clade III, more specifically, widely used insect biocontrol agents in clade III including *B. bassiana*, *M. flavoviride*, and *P. tenuipes*, or the *Ophiocordyceps* genus, and wood-inhabiting clade III. These include QHP and $ERRT_{(S)}H$ for insect-pathogenic fungi versus QRP and QRRSL for wood-inhabiting fungi (Table 4). It would be relevant to test whether site-direct mutagenesis could prove that the conserved regions in these reducing clade III PKSs are essential for the fungi to thrive in those habitats.

Numerous newly cloned PKS-NRPS hybrids in reducing clade II. All the 17 reference clade II PKSs (indicated by "Cl2" at the ends of names in Fig. 1) have the CON-AMP-PP (nonribosomal peptide synthetase [NRPS] module) in addition to the common PKS domains, except for AtLovB and PcMlcA, the PKSs for the statin-like compounds. Nevertheless, these two PKSs have CON and the N terminus of the AMP domains (38). Here, we isolated 38 potential PKS-NRPS hybrids that were clustered with a high bootstrap support (96%) in reducing clade II (Fig. 1 and Table 2). Also, there are 47 more clade II PKSs from fungal genomes (Table 3). Many important secondary metabolites are synthesized by PKS-NRPS hybrids (8). Functional analysis of these PKS genes could be useful to determine whether these PKS enzymes are involved in biosynthesis of valuable target compounds. In addition, the nature of these hybrid compounds, which have both PK and amino acid residues, provides a rich source of diverse secondary metabolite compounds and a possibility for shuffling between PK and amino acids using this pool of PKS-NRPS hybrids.

Similar to the tight clustering of clade III, six newly cloned reducing clade II PKSs are found only in insect-pathogenic

fungi, with 64% bootstrap support: AknF1R1E, AstF1R1B2, CopF1R1B1, CopF1R1F, GibF1R1B, and IsjF1R2D3. These fungi belong to the families *Cordycipitaceae* (five fungi) and *Clavicipitaceae* (one fungus) in *Hypocreales* (Fig. 1). *B. bassiana* TenS, involved in the biosynthesis of tenellin and initially presumed to be involved in insect pathogenesis, was later found to be unnecessary for infection of wax moth larvae (11). Whether this PK/peptide hybrid might be needed in a different stage in this insect or specific for other insects is unclear. However, our phylogenetic analysis also placed TenS outside this insect-specific clade II group.

Distinct clades of reducing fungal PKSs and proposed designation of reducing clade V. Our alignment and phylogenetic analysis discovered four distinct clades. These clades were distinguished from the existing clades (26) as determined by both the NJ and MP analyses, with higher than 75% bootstrap support. The first two novel clades were usually associated with the reducing clade IV (26). The first group (D1) consisted of nine newly cloned PKS genes with a very strong bootstrap support (100%) (BebF1R1A2, CopF1R2E, EpiF1R2B1, GraF1R1C, LirFiR1B2, MicF1R2D, NagF1R1B1, PyrF1R2A1, and XylPKSKA1) as well as one reference PKS, BfPKS10, previously classified as clade IV by Kroken et al. (26) (group D1 in Fig. 1). All members in this group were from marine-derived or lichenized fungi, except for two PKSs from *B. bassiana* and *C. pseudomilitaris*. We propose to designate this monophyletic group reducing clade V. The second group (D2), with 83% bootstrap support, has five members; all were newly cloned with the KA series primers (AstF1R1B, BebF1R2B3, HymdF1R2_J9, PhoF1R2B, and XylF1R1A3).

A group of previously identified clade I PKSs (AtlovFCl1, ChPKS3Cl1, and PcmlcBCl1) were reclassified in our study as the third distinct clade (D3 in Fig. 1). Although they were classified as other clades by Kroken et al. (26), our phylogenetic analysis of the KS-AT region revealed that these PKSs form a distinct group (90% bootstrap support) from the previously identified clades. Clade D3 also included three newly cloned PKSs (AcrF1R2A5, KagF1R1A, and SarF1R1B1) from two marine fungi and one lichenized fungus. Finally, clade D4 contained ChPKS1Cl1, CHPKS7Cl1, and DmPKS1Cl1 (reference PKSs) and CopF1R1XA and LecF1R1B1 (newly cloned PKSs), with 79% bootstrap support. The difference in clade assignment of these PKSs between this study and that of Kroken et al. could be due to the difference in regions used in the phylogenetic analysis: the KS-AT region (this study) versus KS (26). Another reason is perhaps because clades I and IV clustered with only moderate bootstrap support (67 and 61%, respectively, in the study by Kroken et al. [26]) compared with their reducing clades II and III (100 and 97% [26]). In our phylogeny, none of the reference PKSs in clades II and III were classified in different clades from the original classification (26). Likewise, none of the reference PKSs in clades I and IV were placed in clades II and III in our tree.

In addition, one clade, D5, consisted of only two PKSs, AstF1R2A1 and MeaF1R2A1. This clade also included three PKSs from the fungal genomes searched with 100% bootstrap support (see Fig. S1 in the supporting material). Lastly, clade D6 was grouped with 51% bootstrap and was composed of seven members: the PKS gene fragments AcrF1R1B7 and AcrF1R1A12 (from *Acremonium* sp. strain BCC9257), GraF1R2B (*Graphis afzelii*), KagF1R2Y (*Kallichroma glabrum*), LirF1R1A2 (*Lineolata rhizosphorae*), and MasF1R1B (*Massarina* sp. strain BCC18015) and one reference PKS, PKS2 from *Botryotinia fuckeliana* (Fig. 1). Interestingly, these PKSs were mostly from marine fungi, except that there was one PKS from a lichenized fungus.

The finding of new clades, particularly D1 and D2, which are composed of nearly all or all PKS genes that we identified from the tropical fungi is fascinating. It suggests that natural fungal resources in a certain geographical region such as Thailand might be valuable sources for unique PKS enzymes. Duke et al. (10) suggested that the fungi that possess unique PKS genes are more likely to produce unique and interesting PKs. We speculate that the PKSs in these distinct clades might be involved in the biosynthesis of new PK compounds with novel structures. Further investigation of PK products generated by these enzymes should be highly worthwhile.

Distribution and evolution of clade III PKS genes within *Pezizomycotina*. All the insect fungi in this study are from the class *Sordariomycetes* (Table 1). The insect fungal group that has PKSs in clade III is from the order *Hypocreales* (Fig. 3). However, after assembling data from genomic PKSs, two PKSs from *Aspergillus* species were placed within this group, which added members from a different fungal order, *Eurotioles* (class *Eurotiomycete*). Within this fungal class, *A. niger*, *A. oryzae*, and *A. terreus* have PKSs in clade III, whereas *A. clavaus*, *A. fumigatus*, *A. nidulans*, and *Neosartorya fischeri* (a close relative of *A. fumigatus*) do not (Table 3). This division closely followed the taxonomy of this *Aspergillus* group (35). This suggests that clade III PKS genes might have been lost in the *A. clavaus-A. fumigatus-A. nidulans* lineage during the split between these two *Aspergillus* subgroups. Nevertheless, classification of PKSs within this insect-specific branch fairly follows the classical taxonomy, in which these PKSs are remarkably clustered in the families *Cordycipitaceae* and *Ophiocordycipitaceae* (Fig. 3).

In addition, it should be noted that each fungus had only one PKS gene in the reducing clade III. In contrast, in the other three reducing clades some fungi contained more than one PKS gene, for example, *Aschersonia tubulata* (three PKSs in clade II), *Cordyceps pseudomilitalis* (three in II), *Hymenostilbe* sp. strain BCC1571 (three in IV), *Kallichroma glabrum* (three in IV), *Ophiocordyceps unilateralis* (three in IV), *Phomopsis* sp. strain BCC9451 (three in I), and *Xylaria* sp. strain BCC1067 (four in I) (Fig. 1 and Table 2). This multi-PKS presence of each clade in each fungus was even more evident when analyzing fungal genomes. There are 4 (clade I) and 7 (II) PKSs in *A. clavatus*; 10 (I), 6 (II), and 8 (IV) in *A. niger*; and 7 (I) and 4 (II) in *M. grisea* (Table 3). Furthermore, there was evidence of duplicate copies of nearly identical PKS genes in the same fungi in clade IV. Three pairs of very similar PKSs were found in *Colletotrichum gleosporiodes*, *Hymenostilbe* sp., and *Kallichroma glabrum*: CogF1R2B and CogF1R1A1, HymF1R1D and HymF1R1B, and KagF1R1E and KagF1R2E (Fig. 1). These genes might be relatively important for these fungi, as each pair in each fungus may have compensatory functions. These results support the theory of gene duplication in the evolutionary process of PKS family suggested by Kroken et al. (26) and Jenke-Kodama et al. (23). Taking the results together, duplication and divergence may be common in reducing clades

I, II, and IV. In contrast, reducing clade III is perhaps more preserved in the gene evolution process.

Among these 48 fungal isolates in this study, 3 isolates have been reported to produce novel compounds and/or have biological activities. These are *Cordyceps nipponica*, *C. pseudomilitaris*, and *Paecilomyces tenuipes*. *C. nipponica* BCC1389 produces cordypyridones A to D, and the isomers A and B have strong antimalarial activity (20). It is noted that this isolate of *C. nipponica* also has two PKS-NRPS hybrids, CniF1R1E and CniF1R2A, in reducing clade II, which are adjacent to *B. bassiana* TenS, which is involved in biosynthesis of another 2-pyridone tenellin in our phylogentic tree (Fig. 1). We perhaps should make no further speculation about the link between the two *C. nipponica* PKS-NRPS genes found in this study and the pyridines produced from this fungus. However, this study identifies a potential correlation between our genetic data and the biochemical data described previously. Targeted disruption of the two hybrid genes would determine their involvement in biosynthesis of cordypyridones A to D in *C. nipponica*. Two other fungi screened in this study also produce bioactive compounds. *C. pseudomilitaris* BCC1620 produces 11 bioxanthracenes and two monomers, with antimalarial activity (22), whereas *P. tenuipes* BCC1614 produces three cyclohexadepsipeptides, beauvericins A to C (32).

In summary, we identified a potential and diverse collection of PKS genes from our tropical fungi. Unique and intriguing relationships between the fungal enzymes, the fungal habitats, and the fungal natural products were observed and discovered. These are subjects of our ongoing and future studies to determine whether experimental evidence would support our hypotheses.

ACKNOWLEDGMENTS

We are grateful to Lynn Epstein, Sonia Herrero, and Jariya Sakayaroj for critical reading of the manuscript and to Juntira Punya and Suparat Pinsupa for help and advice in conducting experiments.

REFERENCES

1. **Alves, S. B., M. A. Tamai, L. S. Rossi, and E. Castiglioni.** 2005. *Beauveria bassiana* pathogenicity to the citrus rust mite *Phyllocoptruta oleivora*. Exp. Appl. Acarol. **37:**117–122.
2. **Amnuaykanjanasin, A., J. Punya, P. Paungmuang, A. Rungrod, A. Tachaleat, S. Pongpattanakitshote, S. Cheevadhanarak, and M. Tanticharoen.** 2005. Diversity of type I polyketide synthase genes in the wood-decay fungus *Xylaria* sp. BCC 1067. FEMS Microbiol. Lett. **251:**125–136.
3. **Baker, S. E.** 2006. *Aspergillus niger* genomics: past, present and into the future. Med. Mycol. **44**(Suppl. 1)**:**S17–S21.
4. **Bingle, L. E., T. J. Simpson, and C. M. Lazarus.** 1999. Ketosynthase domain probes identify two subclasses of fungal polyketide synthase genes. Fungal Genet. Biol. **26:**209–223.
5. **Böhnert, H. U., I. Fudal, W. Dioh, D. Tharreau, J. L. Notteghem, and M. H. Lebrun.** 2004. Putative polyketide synthase/peptide synthetase from *Magnaporthe grisea* signals pathogen attack to resistant rice. Plant Cell **16:**2499–2513.
6. **Bok, J. W., L. Lermer, J. Chilton, H. G. Klingeman, and G. H. N. Towers.** 1999. Antitumor sterols from the mycelia of *Cordyceps sinensis*. Phytochemistry **51:**891–898.
7. **Calvo, A. M., R. A. Wilson, J. W. Bok, and N. P. Keller.** 2002. Relationship between secondary metabolism and fungal development. Microbiol. Mol. Biol. Rev. **66:**447–459.
8. **Collemare, J., A. Billard, H. U. Böhnert, and M. H. Lebrun.** 2008. Biosynthesis of secondary metabolites in the rice blast fungus *Magnaporthe grisea*: the role of hybrid PKS-NRPS in pathogenicity. Mycol. Res. **112:**207–215.
9. **Dean, R. A., et al.** 2005. The genome sequence of the rice blast fungus *Magnaporthe grisea*. Nature **434:**980–986.
10. **Duke, S. O., et al.** 2003. United States Department of Agriculture-Agricultural Research Service research on natural products for pest management. Pest Manag. Sci. **59:**708–717.
11. **Eley, K. L., L. M. Halo, Z. Song, H. Powles, R. J. Cox, A. M. Bailey, C. M. Lazarus, and T. J. Simpson.** 2007. Biosynthesis of the 2-pyridone tenellin in the insect pathogenic fungus *Beauveria bassiana*. Chembiochem **8:**289–297.
12. **Espagne, E., et al.** 2008. The genome sequence of the model ascomycete fungus *Podospora anserina*. Genome Biol. **9:**R77.
13. **Essien, J. P.** 2004. Insecticidal potential of an orally administered metabolic extract of *Aspergillus niger* on *Chrysomya chloropyga* (green bottle fly) larvae. J. Appl. Sci. Environ. Manag. **8:**45–48.
14. **Fedorova, N. D., et al.** 2008. Genomic islands in the pathogenic filamentous fungus *Aspergillus fumigatus*. PLoS Genet. **4:**e1000046.
15. **Gaffoor, I., D. W. Brown, R. Plattner, R. H. Proctor, W. Qi, and F. Trail.** 2005. Functional analysis of the polyketide synthase genes in the filamentous fungus *Gibberella zeae* (anamorph *Fusarium graminearum*). Eukaryot. Cell **4:**1926–1933.
16. **Galagan, J. E., et al.** 2005. Sequencing of *Aspergillus nidulans* and comparative analysis with *A. fumigatus* and *A. oryzae*. Nature **438:**1105–1115.
17. **Hall, T. A.** 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Res. Symp. Ser. **41:**95–98.
18. **Hane, J. K., et al.** 2007. Dothideomycete plant interactions illuminated by genome sequencing and EST analysis of the wheat pathogen *Stagonospora nodorum*. Plant Cell **19:**3347–3368.
19. **Hywel-Jones, N. L.** 2001. A review of invertebrate pathogenic Clavicipitaceae of Thailand, p. 34–41. *In* V. Baimai and K. Rungsin (ed.), BRT research reports 2001. Jirawat Express Co., Bangkok, Thailand.
20. **Isaka, M., M. Tanticharoen, P. Kongsaeree, and Y. Thebtaranonth.** 2001. Structures of cordypyridones A–D, antimalarial *N*-hydroxy- and *N*-methoxy-2-pyridones from the insect pathogenic fungus *Cordyceps nipponica*. J. Org. Chem. **66:**4803–4808.
21. **Jacobs, M., and U. Stahl.** 1995. Gene regulation in mycelial fungi, p. 155–167. *In* U. Kueck (ed.), The Mycota. II. Genetics and biotechnology. Springer-Verlag, New York, NY.
22. **Jaturapat, A., M. Isaka, N. L. Hywel-Jones, Y. Lertwerawat, S. Kamchonwongpaisan, K. Kirtikara, M. Tanticharoen, and Y. Thebtaranonth.** 2001. Bioxanthracenes from the insect pathogenic fungus. Cordyceps pseudomilitaris BCC 1620. I. Taxonomy, fermentation, isolation and antimalarial activity. J. Antibiot. **54:**29–35.
23. **Jenke-Kodama, H., A. Sandmann, R. Müller, and E. Dittmann.** 2005. Evolutionary implications of bacterial polyketide synthases. Mol. Biol. Evol. **22:**2027–2039.
24. **Ji, D. B., J. Ye, C. L. Li, Y. H. Wang, J. Zhao, and S. Q. Cai.** 2009. Antiaging effect of *Cordyceps sinensis* extract. Phytother. Res. **23:**116–122.
25. **Khosla, C., R. S. Gokhale, J. R. Jacobsen, and D. E. Cane.** 1999. Tolerance and specificity of polyketide synthases. Annu. Rev. Biochem. **68:**219–253.
26. **Kroken, S., N. L. Glass, J. W. Taylor, O. C. Yoder, and B. G. Turgeon.** 2003. Phylogenomic analysis of type I polyketide synthase genes in pathogenic and saprobic ascomycetes. Proc. Natl. Acad. Sci. USA **100:**15670–15675.
27. **Lee, T., S. H. Yun, H. T. Hodge, R. A. Humber, S. B. Krasnoff, G. B. Turgeon, O. C. Yoder, and D. M. Gibson.** 2001. Polyketide synthase genes in insect- and nematode-associated fungi. Appl. Microbiol. Biotechnol. **56:**181–187.
28. **Liou, G. F., and C. Khosla.** 2003. Building-block selectivity of polyketide synthases. Curr. Opin. Chem. Biol. **7:**279–284.
29. **Mayer, K., J. Ford, G. Macpherson, D. Padgett, B. Volkmann-Kohlmeyer, J. Kohlmeyer, C. Murphy, S. Douglas, J. Wright, and J. L. C. Wright.** 2007. Exploring the diversity of marine-derived fungal polyketide synthases. Can. J. Microbiol. **53:**291–302.
30. **Machida, M., et al.** 2005. Genome sequencing and analysis of *Aspergillus oryzae*. Nature **438:**1157–1161.
31. **Nierman, W. C., et al.** 2005. Genomic sequence of the pathogenic and allergenic filamentous fungus *Aspergillus fumigatus*. Nature **438:**1151–1156.
32. **Nilanonta, C., M. Isaka, P. Kittakoop, P. Palittapongarnpim, S. Kamchonwongpaisan, D. Pittayakhajonwut, M. Tanticharoen, and Y. Thebtaranonth.** 2000. Antimycobacterial and antiplasmodial cyclodepsipeptides from the insect pathogenic fungus *Paecilomyces tenuipes* BCC 1614. Planta Med. **66:**756–758.
33. **O'Hagan, D.** 1991. Fungal and plant polyketides, p. 65–110. *In* D. O'Hagan (ed.), The polyketide metabolites. Ellis Howard, New York, NY.
34. **Peveling, R., and S. A. Demba.** 1997. Virulence of the entomopathogenic fungus *Metarhizium flavoviride* Gams and Rozsypal and toxicity of diflubenzuron, fenitrothion-esfenvalerate and profenofos-cypermethrin to nontarget arthropods in Mauritania. Arch. Environ. Contam. Toxicol. **32:**69–79.
35. **Pontecorvo, G., J. A. Roper, L. M. Hemmons, K. D. Macdonald, and A. W. Bufton.** 1953. The genetics of *Aspergillus nidulans*. Adv. Genet. **5:**141–238.
36. **Reader, U., and P. Broda.** 1985. Rapid preparation of DNA from filamentous fungi. Lett. Appl. Microbiol. **1:**17–20.
37. **Reeves, C. D., Z. Hu, R. Reid, and J. T. Kealey.** 2008. Genes for the biosynthesis of the fungal polyketides hypothemycin from *Hypomyces subiculosus* and radicicol from *Pochonia chlamydosporia*. Appl. Environ. Microbiol. **74:**5121–5129.
38. **Sims, J. W., J. P. Fillmore, D. D. Warner, and E. W. Schmidt.** 2005. Equisetin biosynthesis in *Fusarium heterosporum*. Chem. Commun. **2:**186–188.

39. **Suzuki, K.** 1995. Biological control of termites by pathogenic fungi, p. 146–156. *In* Proceedings of the Third Conference on Forestry and Forest Products Res. Forest Research Institute, Malaysia.
40. **Swofford, D. L.** 2002. PAUP*: phylogentic analysis using parsimony (*and other methods), version 5. Sinauer Associates, Sunderland, MA.
41. **Thompson, J. D., T. J. Gibson, F. Plewniak, F. Jeanmougin, and D. G. Higgins.** 1997. The CLUSTAL-X Windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res. **25:**4876–4882.
42. **Yamaguchi, Y., S. Kagota, K. Nakamura, K. Shinozuka, and M. Kunitomo.** 2000. Antioxidant activity of the extracts from fruiting bodies of cultured *Cordyceps sinensis*. Phytother. Res. **14:**647–649.