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Amplification and diversity analysis of ketosynthase domains of putative polyketide synthase genes in *Aspergillus ochraceus* and *Aspergillus carbonarius* producers of ochratoxin A

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The diversity of polyketide synthase (PKS) genes in *Aspergillus ochraceus* NRRL 3174 and *Aspergillus carbonarius* 2Mu134 has been investigated using different primer pairs previously developed for the ketosynthase (KS) domain of fungal PKSs. Nine different KS domain sequences in *A. ochraceus* NRRL 3174 as well as five different KS domain sequences in *A. carbonarius* 2Mu134 have been identified. The identified KS fragments were distributed in five different clusters on the phylogenetic tree, indicating that they most probably represent PKSs responsible for different functions.

Keywords: *Aspergillus carbonarius* / *Aspergillus ochraceus* / Mycotoxins / Ochratoxin A / Polyketide synthase

1 Introduction

Fungal polyketide synthases (PKSs) are responsible for the biosynthesis of several mycotoxins and other secondary metabolites through sequential decarboxylative condensations of short carboxylic acid units similarly to fatty acid synthases [1, 2]. Recently, several genomes of filamentous fungi have been sequenced. The sequencing data revealed a large number of putative PKS genes existing in filamentous fungi [3–6]. These fungal PKS genes outnumber those found in the genomes of *Streptomyces avermitilis*, *Streptomyces coelicolor*, and actinomycete species that are traditionally regarded as the most abundant source for polyketide metabolites [3].

Fungal PKSs are classified into three groups based on the molecular architecture of the genes: (i) PKSs for nonreduced polyketides, (ii) PKSs for partially reduced polyketides, and (iii) PKSs for reduced polyketides [1, 7]. They are defined as iterative type I PKSs because they usually

contain only a single set of domains, in contrast to the non-iterative modular type I PKSs and monofunctional type II PKSs found in bacteria [7–9]. Typically, a module consists of a β -ketoacyl synthase, an acyltransferase (AT), and an acyl carrier protein. Many PKSs, especially those for reduced polyketides, also contain additional domains, such as β -ketoacyl reductase, dehydratase, enoylreductase, and methyltransferase [7]. A single-modular PKS can catalyze multiple cycles of chain elongation [1, 8, 10].

Among the mycotoxigenic fungi, *Aspergillus ochraceus* and *Aspergillus carbonarius* are considered as the most frequent fungal contaminants of several agricultural products. *A. ochraceus* is able to contaminate many food commodities including cereals, coffee, grapes, and others, often producing several mycotoxins such as ochratoxin A (OTA), viomellein, and penicillic acid [1]. *A. carbonarius* is considered to be responsible for OTA contamination in grapes, wines, and coffee [11]. Both species are also capable of producing other polyketides such as naphthopyrones, mellein, and hydroxymellein.

Until now, five PKS genes have been identified in *A. ochraceus* including four ketosynthase (KS) domain sequences [1, 12] and one AT domain sequence [13]. However, no PKS gene has been identified in *A. carbonarius*.

In this work, we used a PCR approach including different sets of degenerated and specific primers targeting the KS domain in order to investigate the diversity of PKS genes in *A. ochraceus* NRRL 3174 and *A. carbonarius* 2Mu134.

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Abbreviations: AT, acyltransferase; Blastp, protein-protein Blast; KS, ketosynthase; MSAS, 6-methylsalicylic acid synthase; OTA, ochratoxin A; PKS, polyketide synthase

Obtained KS domain sequences were subjected to phylogenetic analysis to predict their functional role in both species. Kinetic expression analysis of *AoLC35-2* sequence, which was thought to be involved in penicillic acid biosynthesis, was performed.

2 Materials and methods

2.1 Fungal strains and culture conditions

A. ochraceus NRRL 3174 and *A. carbonarius* 2Mu134, which was isolated from French vineyards, were grown for sporulation at 25°C on potato dextrose agar (Difco, Fisher Labosi) for 7 days. Spores were collected using a solution of 0.01% v/v Tween 80 (Fisher Labosi), and stored at -20°C in 25% v/v glycerol (Fisher Labosi) before use. Conidia (final concentration of 10⁶ spores/mL) were inoculated into 250 mL Erlenmeyer flasks containing 100 mL of synthetic medium at 25°C, without shaking. Synthetic medium was prepared according to Awad *et al.* [14]. Mycelium was harvested by filtration through a 0.45 µm filter (Millipore), frozen in liquid nitrogen, and then stored at -80°C before nucleic acid extraction. Filtered medium was used to extract secondary metabolites. For dry weight determination, mycelium was dried overnight in oven at 100°C.

2.2 Extraction of penicillic acid and HPLC analysis

For penicillic acid extraction, 30 mL of filtrate samples were acidified with 200 µL of 12 N HCl, mixed with 20 mL chloroform. The organic phase was taken and concentrated under vacuum to dryness, and the residue was redissolved in 0.5 mL of methanol. A volume of 80 µL of the sample was analyzed by HPLC to detect penicillic acid as described by Awad *et al.* [14].

2.3 Nucleic acid extraction

Genomic DNA was extracted by using lysing enzyme and proteinase K as described previously [15]. Total RNA was extracted from *A. ochraceus* NRRL 3174 using the Tri-reagent (Euromedex) which included the following steps: An amount of 200 mg of frozen mycelia was homogenized in 1 mL of Tri-reagent by using an Ultra-Turax. After 5 min at room temperature, 0.2 mL of chloroform was added to the homogenized samples which were vigorously vortexed, stored for 10 min at room temperature, and then centrifuged at 15 000 × g for 15 min at 4°C. The aqueous phase containing RNA was transferred into a microcentrifuge tube, and then 0.5 mL isopropanol (Euromedex) was added for RNA precipitation at room temperature (15 min). The RNA was pelleted by centrifuging at 12 000 × g for 15 min at 4°C,

washed with 1 mL of 75% ethanol, then air-dried, and dissolved in 50 µL of 0.1% diethylpyrocarbonate (Sigma) water. The quality and quantity of RNA were checked by the OD_{260/280} ratio and agarose (Fisher Labosi) gel electrophoresis according to standard protocols [16].

2.4 cDNA synthesis

Total RNA was treated with DNase I (Promega) to remove DNA contamination. cDNA was synthesized from each sample with Advantage RT-for-PCR Kit (BD Biosciences) according to the user manual provided.

2.5 PCR reaction and sequencing

PCR was performed with the *Taq* recombinant polymerase (Invitrogen, USA). Amplification was carried out in 50 µL of reaction mixture containing: 5 µL of *Taq* polymerase 10^x buffer, 1.5 µL of 50 mM MgCl₂, 1 µL of dNTP 10 mM of each (Promega), 1 µM of each primer, 1.5 u of *Taq*, about 100 ng of genomic DNA, and H₂O up to 50 µL. Reaction conditions were: 94°C for 4 min, (94°C for 45 s, 50°C for 45 s, and 72°C for 45 s) × 30 cycles followed by an incubation at 72°C for 10 min. The amplified products were examined by 1% w/v agarose (Promega) gel. The PCR products were cloned into TOPO TA Cloning® vector (Invitrogen) according to the supplier's instructions. Sequencing reactions were performed by Genomexpress (Grenoble, France).

2.6 Data analysis

The deduced amino acid sequence was determined using the <http://www.expasy.org/tools/dna.html> site while protein-protein Blast (Blastp) searches were conducted at the GenBank database: <http://www.ncbi.nlm.nih.gov/>. The alignments were conducted using the website <http://prodes.toulouse.inra.fr/multalin/multalin.html>. Intron in DNA sequence was predicted by the presence of canonical GT and AG dinucleotides. For the phylogenetic tree construction, 152 amino acids of our different identified KS domains and those found by Blastp searches were analyzed using the ClustalX package version 1.83 [17] and MEGA2 package [18]. Unroot consensus tree was generated using neighbor-joining method.

2.7 Sequence accession number

The nucleotide sequences have been deposited in the GenBank database with the accession numbers provided in Table 1.

Table 1. Putative PKSs identified in this study

Sequences	Species	Primer pair used	Accession number
<i>AcKS9</i>	<i>A. carbonarius</i>	KS1/KS2	AY540944
<i>AoKS1</i>	<i>A. ochraceus</i>	KS1/KS2	AY583209
<i>AoKS11</i>	<i>A. ochraceus</i>	KS1/KS2	AY540950
<i>AoLC35-6</i>	<i>A. ochraceus</i>	LC3/LC5c	AY540951
<i>Ac12RL3</i>	<i>A. carbonarius</i>	AoLC35-12L/R	AY652734
<i>AoKS9</i>	<i>A. ochraceus</i>	KS1/KS2	AY583207
<i>AcKS10</i>	<i>A. carbonarius</i>	KS1/KS2	AY540952
<i>AoLC35-12</i>	<i>A. ochraceus</i>	LC3/LC5c	AY583208
<i>AoLC35-2</i>	<i>A. ochraceus</i>	LC3/LC5c	AY540947
<i>AcLC35-4</i>	<i>A. carbonarius</i>	LC3/LC5c	DQ128160
<i>AcLC35-6</i>	<i>A. carbonarius</i>	LC3/LC5c	DQ128161
<i>AoLC12-9</i>	<i>A. ochraceus</i>	LC1/LC2c	AY540945
<i>AoLC12-12</i>	<i>A. ochraceus</i>	LC1/LC2c	AY540946
<i>AoLC12-14</i>	<i>A. ochraceus</i>	LC1/LC2c	AY540949

3 Results and discussion

3.1 Amplification and analysis of KS domain sequences

Using different degenerated and specific primer pairs based on the conserved KS domain of PKSs, nine different KS domain sequences in *A. ochraceus* NRRL 3174 and five different KS domain sequences in *A. carbonarius* 2Mu134 have been obtained (Table 1, Fig. 1). The conserved pattern D(T/A)AC(S/A)(S/A/G)S characteristic of the KS domain was displayed in each sequence (Fig. 1).

With the primer pair KS1/KS2 designed to target fungal and bacterial KS domains [10], three different KS sequences in *A. ochraceus* (*AoKS1*, *AoKS9*, and *AoKS11*) as well as two different KS sequences in *A. carbonarius* (*AcKS9* and *AcKS10*) were identified (Table 1, Fig. 1). By Blastp analysis, amino acid sequences of *AoKS1*, *AoKS11*, and *AcKS9* displayed about 60% identity to nonaketide synthases involved either in lovastatin biosynthesis (lovB) in *A. terreus* [19], or in compactin biosynthesis (mlcA) in *Penicillium citrinum* [20]. Sequences *AoKS9* and *AcKS10*, carrying an intron of 65 and 50 bp, respectively, their

deduced protein sequences were mostly related (52% identity) to diketide synthases involved either in lovastatin biosynthesis (lovF) in *A. terreus*, or in compactin biosynthesis (mlcB) in *P. citrinum*. Additionally, *AcKS10* displayed about 60% identity to *AoLC35-12* (Table 1) which was found to join AT domain sequence of the PKS involved in OTA biosynthesis in *A. ochraceus* [13].

Using the primer pair LC3/LC5c targeting 6-methylsallylic acid synthase (MSAS)-type PKSs [9, 21]; three different KS sequences in *A. ochraceus* (*AoLC35-2*, *AoLC35-6*, and *AoLC35-12*) and two different KS sequences in *A. carbonarius* (*AcLC35-4* and *AcLC35-6*) were amplified and sequenced (Table 1, Fig. 1). *AoLC35-12* was found to carry an intron of 66 bp while *AoLC35-2*, *AoLC35-6*, *AcLC35-4*, and *AcLC35-6* contained none. *AoLC35-2* amino acid sequence displayed about 68 and 60% identity to PKSs responsible for MSAS in *Byssoschlamys nivea* and *A. terreus*, respectively. *AoLC35-6* showed 63% identity with the nonaketide synthases involved in the biosynthesis of lovastatin in *A. terreus* or compactin in *P. citrinum*. However, this sequence is totally different from *AoKS11* and *AoKS1*. *AoLC35-12* displayed about 42% identity to diketide synthases in *A. terreus* (lovF) or *P. citrinum* (mlcB).

AoLC35-12 sequence was previously described in our earlier publication [15], and it was found to be the KS domain of the aopks sequence (Genbank accession number AY272043) involved in OTA biosynthesis in *A. ochraceus* [13].

AcLC35-4 displayed about 47% identity to PKSs responsible for MSAS in *Aspergillus terreus* and *P. patulum*, and 44% identity to OTA PKS (*otapks*PN, Genbank accession number AY196315) in *P. nordicum* [21]. *AcLC35-6* was found to have 47% identity to the PKS involved in MSAS in *B. nivea*. Furthermore, two sequences *AoKS1* and *AoKS11* in *A. ochraceus* and one sequence *AcKS10* in *A. carbonarius* obtained by the primer pair KS1/KS2 were reamplified by the primer pair LC3/LC5c.

With the primer pair LC1/LC2c amplifying nonreduced or WA-type PKSs [9, 22], three different KS sequences in *A.*

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Ac12RL3  GTSQTIDTACSSALTLHLAAREALQNGDCSL-AYAAGSSLILSPDPYIGESQMQLSPTGR
AoKS11  GPSLYLDTACSSSLIALHLAVQALQKGDSCM-AYAAGSSLILSVGYYSSSQANMSPSGR
AoKS1   GPSMTIDTACSSSLVALHLAAKALHDGECRY-VYASGTNLILAPNHYISEKLSMLSPHGR
AcKS9   GPSMTIDTACSSSLVALHQGVGALRSGDCHL-AYIAASNLIFSPREYIASKMHLLSPTGR
AcKS10  GPSVYVDTACSGSLVALHLACQSLRTGDASH-AYAAGVNVILSHEFMSTMTMHKFLSPNGR
AoLC35-6  GTSQTIDTACSSSLVAFHQACHDVRTGRSPM-SVYSGVNLIEHPAATLYLSNLGVLSPDGR
AoLC35-12 GTSQTIDTACSSSLVAFHQACHDVRTGRSPM-SVYSGVNLIEHPAATLYLSNLGVLSPDGR
AcLC35-4  GPSEPIDTACSSSLVAIHHAHVSSIEEGTCDM-ALAGGINTIYVLPDYYISFDKAGALSKEGR
AcLC35-6  GPSIAYDTACSSSLTRAIHLAESIRTGASEC-AIAGGVNLTDPYHYIGLSSMRHLSPSNA
AoLC35-2  GPSVSYDAACASSLVAVHNGVQAILLEGESTY-VIAGGVHAMCGPALTRYMDKAGVLPDGR
AoLC12-9  GPSYSVDTACSSSLASLHVACNALWRGDIDT-AIAGGTNVLTPDFTAGLDRGHFLSRTGN
AoLC12-12 GPSLSIDTACSSSFARAIQTACAYLWRGECDT-ALAGGVNMTNPNDFAGLDRGHFLTTKGN
AoLC12-14 GPSYSIDAACSSSMARIIQIGLTALRSRECDM-TVAGGANIMTSSDPFAGLSRGSFLSPTGS
AoKS9   GPSATIDTACSSSLVCFHMGQSLRAGEADLFCRPGPALHFDPNIFITHTDLGMLSTDGR
Consensus GpS...!DtACSS$*va.h.a...lr.g.....a.a.G.nli..p.....Lsp.Gr

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Figure 1. KS domain alignment of the identified putative PKS sequences.

ochraceus were obtained: *AoLC12-9*, *AoLC12-12*, and *AoLC12-14* (Table 1, Fig. 1). *AoLC12-12* DNA sequence was found to carry two introns of 54 and 48 bp. *AoLC12-14* contained one of 50 bp and *AoLC12-9* none. Blastp analysis showed, for *AoLC12-9*, 86% identity to amino acid sequences encoded by *pks3* (unknown function) from *Gibberella fujikuroi* and *G. moniliformis* [23], and for *AoLC12-12*, 80% identity to *at4* in *A. terreus* that was required in naphthopyrone biosynthesis. *AoLC12-14* exhibited about 60% identity to PKSs responsible for melanin biosynthesis in *Nodulisporium* sp. [24] and *Colletotrichum lagenarium* [25]. No amplification was carried out in *A. carbonarius* with this primer pair because our strategy was to investigate the diversity of the reduced and partially reduced PKSs types in this fungus in order to identify the PKS responsible for OTA biosynthesis.

Finally, using the specific primer pair *AoLC35-12L/AoLC35-12R* deduced from the conserved motifs of the KS domain of the PKS gene involved in OTA biosynthesis in *A. ochraceus* [15], one sequence was obtained in *A. carbonarius*, *Ac12RL3* (Table 1, Fig. 1). By Blastp analysis, amino acid sequence of *Ac12RL3* displayed about 65% identity to nonaketide synthases involved in lovastatin biosynthesis in *A. terreus* or in compactin biosynthesis in *P. citrinum*.

The result of Blastp allows us to divide the obtained sequences in three different groups; the first group contained sequences (*AoKS1*, *AoKS9*, *AoKS11*, *Ac12RL3*, *AcKS9*, and *AcKS10*) which were found to be closely related to the PKSs involved in reduced polyketides biosynthesis such as compactin, lovastatin, and T-toxin. The second group contained PKS sequences (*AoLC35-2*, *AoLC35-6*, *AoLC35-12*, *AcLC35-4*, and *AcLC35-6*) which were found to be related to the PKSs responsible for partially reduced polyketides biosynthesis such as MSAS. The third group contained sequences (*AoLC12-9*, *AoLC12-12*, and *AoLC12-14*) which were found to be closely related to the PKSs responsible for nonreduced polyketides biosynthesis such as melanins, naphthopyrones, and spore pigments.

Several mycotoxins produced by *A. ochraceus* (such as OTA, penicillic acid, viomellein, and xanthomegnin) and *A. carbonarius* (OTA) belong to the reduced and partially reduced polyketide groups; we are interested in the PKS gene sequences catalyzing the biosynthesis of those groups of polyketides. For example, kinetic study of penicillic acid production and expression of *AoLC35-2* (MSAS type) were performed during the growth of *A. ochraceus* (Fig. 2). Expression of *AoLC35-2* sequence started at day 3, peaked at day 8, and then stopped after day 11. It seems to be coherent with penicillic acid production which started at day 3 and reached the maximum at day 11. These results suggest a link between the function of *AoLC35-2* gene and penicil-

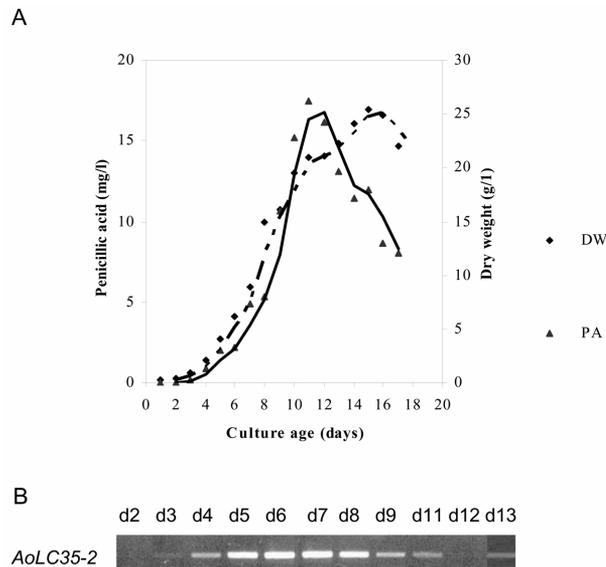


Figure 2. Kinetic of penicillic acid production during growth of *A. ochraceus* (DW) at 25°C in synthetic medium (A) and expression of *AoLC35-2* sequence by RT-PCR (B).

lic acid metabolite produced in *A. ochraceus*. However, further studies are in progress to clarify the role of this gene.

3.2 Clustering of putative PKSs identified

The 14 amplified KS domain sequences obtained in this study have been subjected to phylogenetic analysis together with other fungal PKSs from the database (Fig. 3). Our sequences were highly diverse; they have been found to be distributed on the phylogenetic tree into three main clades as previously defined by several authors [1, 22]: highly reduced PKSs (*AoKS1*, *AoKS11*, *AoKS9*, *AoLC35-6*, *AoLC35-12*, *AcKS9*, *AcKS10*, and *Ac12RL3*), partially reduced PKSs (*AoLC35-2*, *AcLC35-4*, and *AcLC35-6*), and nonreduced PKSs (*AoLC12-9*, *AoLC12-12*, and *AoLC12-14*) (Fig. 3).

In the highly reduced clades, *AoKS1*, *AoKS11*, *AoLC35-6*, *AcKS9*, and *Ac12RL3* were found to be closely related to nonaketide synthases while *AoKS9*, *AoLC35-12*, and *AcKS10* were found to fall in the diketide synthases group (Fig. 3). They formed a lovastatin/T-toxin cluster [1, 9, 23].

In the partially reduced clade, one sequence of *A. ochraceus* NRRL 3174 (*AoLC35-2*) and two sequences of *A. carbonarius* 2Mu134 (*AcLC35-4* and *AcLC35-6*) were closed to PKSs in MSAS cluster (Fig. 3).

All sequences from LC1/LC2 were found to fall into the nonreduced clade (Fig. 3). *AoLC12-14* was closed to PKSs

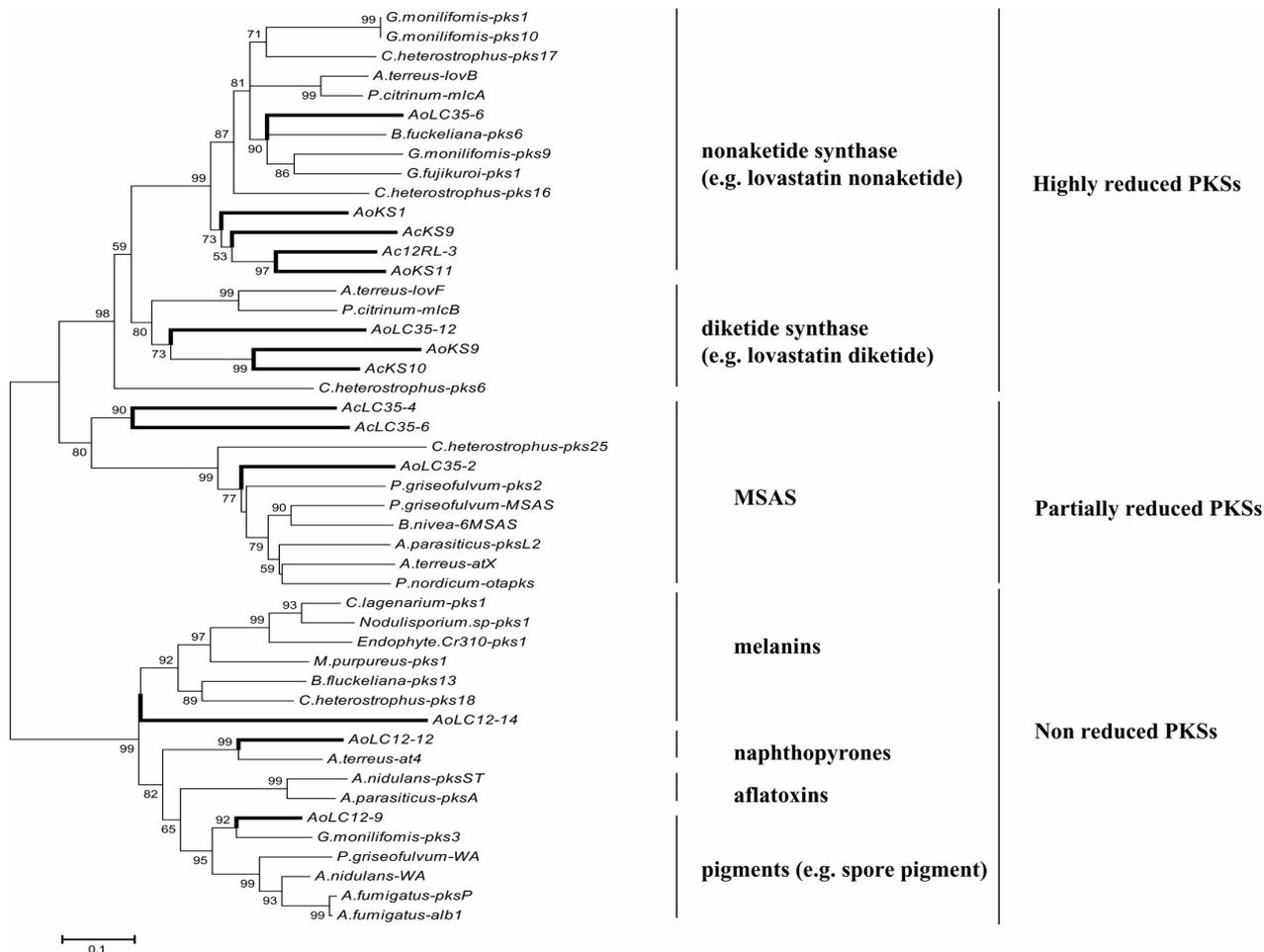


Figure 3. Neighbor-joining phylogenetic tree of KS sequences of fungal PKSs. Bootstrap values above 50 are shown on branches. Fungal PKS clusters are indicated on the right. Abbreviation and accession numbers of PKS sequences used: *A. fumigatus*: pksP (CAA76740), alb1 (AAC39471.1); *A. terreus*: lovB (AAD39830.1), lovF (AAD34559.1), atX (BAA20102.1), at4 (BAB88689.1); *A. parasiticus*: pksL2 (AAC23536.1), PksA (Z47198); *A. nidulans*: WA (CAA46695.2), pksST (AA81586); *Botryotinia fuckeliana*: pks6 (AAR90242.1), pks13 (AAR90249.1); *B. nivea*: MSAS (AAK48943.1); *Cochliobolus heterostrophus*: pks6 (AAR90261.1), pks16 (AAR90270.1), pks17 (AAR90271.1), pks18 (AAR90272.1), pks25 (AY495666); *Colletotrichum lagenarium*: pks1 (BAA18956.1); *Gibberella fujikuroi*: pks1 (CAC44633.1), pks3 (CAC88775.1); *Gibberella moniliformis*: pks1 (AAR92217.1), pks3 (AAR92210.1), pks9 (AAR92216), pks10 (AAR92217.1); *Monascus purpureus*: pks1 (CAC94008.1); *Nodulisporium* sp.: pks1 (AAD38786.1); *P. citrinum*: mlcA (BAC20564.1), mlcB (BAC20566.1); *P. griseofulvum*: pks2 (AAB49684.1), MSAS (S13178), WA (CAB44713); *P. nordicum*: otapksPN (AAP33839); and fungal endophyte sp.: Cr310 (AAP68698).

in melanin cluster, *AoLC12-12* was found in the naphthopyrone cluster while *AoLC12-9* was found in the cluster of PKSs responsible for spore pigments (Fig. 3).

The diversity of the obtained KS domain sequences reflected the diversity of polyketide metabolites produced in both species. *A. ochraceus* is able to produce more than 20 different polyketides [1]. However, in total 14 PKS genes were identified in this study, by Varga *et al.* [1] and Edwards *et al.* [12]. In *A. carbonarius* 2Mu134, five different PKS genes were identified in this study using primer pairs amplifying the reduced-type PKS.

4 Concluding remarks

In this study, we have obtained nine different KS domain sequences in *A. ochraceus* NRRL 3174 and five different KS domain sequences in *A. carbonarius* 2Mu134. Blast and phylogenetic analysis results showed that these sequences were highly diverse indicating that they represent PKSs responsible for different functions. Recently Geisen *et al.* [21] and O'Callaghan *et al.* [13] described part of a gene coding for a PKS involved in OTA biosynthesis in *P. nordicum* and *A. ochraceus*, respectively. The identification of a number of reduced and partially reduced-type PKS

gene sequences in this study will hopefully lead to the identification of several mycotoxins biosynthetic genes in both microorganisms, such as penicillic acid and viomellein in *A. ochraceus*, and OTA in *A. carbonarius*. Consequently, these sequences could help us for the development of specific primers which may be used for the direct detection and quantification of both mycotoxigenic species in a variety of food products.

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