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Exploring the Distribution of Citrinin Biosynthesis Related Genes among *Monascus* Species

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Citrinin, a hepato-nephrotoxic compound to humans, can be produced by the food fermentation microorganisms *Monascus* spp. In this study, we investigated the distribution of mycotoxin citrinin biosynthesis genes in 18 *Monascus* strains. The results show that the acyl-transferase and keto-synthase domains of the *pksCT* gene encoding citrinin polyketide synthase were found in *Monascus purpureus*, *Monascus kaoliang*, and *Monascus sanguineus*. Furthermore, the *ctnA* gene, a major activator for citrinin biosynthesis, was found in *M. purpureus* and *M. kaoliang*, but was absent in *M. sanguineus*. The *orf3* gene encoding oxygenase, located between *pksCT* and *ctnA*, was also present in *M. purpureus* and *M. kaoliang*. The *pksCT* gene was highly conserved in *M. purpureus*, *M. kaoliang*, and *M. sanguineus*, while the *ctnA* and *orf3* genes were shown to be highly homologous in *M. purpureus* and *M. kaoliang*. In contrast, the PCR and Southern blot analyses suggest that *pksCT*, *ctnA*, and *orf3* were absent or significantly different in *Monascus pilosus*, *Monascus ruber*, *Monascus barkeri*, *Monascus floridanus*, *Monascus lunisporas*, and *Monascus pallens*. A citrinin-producing phenotype was detected only in *M. purpureus* and *M. kaoliang* using high performance liquid chromatography (HPLC). These results clearly indicate that the highly conserved citrinin gene cluster in *M. purpureus* and *M. kaoliang* carry out citrinin biosynthesis. In addition, according to the phylogenetic subgroups established with the β-tubulin gene, the citrinin gene cluster can group the species of *Monascus*.

KEYWORDS: Citrinin; *Monascus*; polyketide; β-tubulin

INTRODUCTION

Typically used in fermented foods, the filamentous fungi *Monascus* spp. are known to produce various secondary metabolites with polyketide structures such as pigments, monacolin K, and citrinin (*1*−*3*). The red pigments produced by the various species of *Monascus* have been used extensively as natural food colorants (*4*). Known as lovastatin and normally used for reducing serum cholesterol levels in humans, monacolin K was first isolated from the *Monascus ruber* medium (*1*), although it can also be found in *Aspergillus terreus* (*5*−*7*). Citrinin, the hepato-nephrotoxic agent and Gram-positive bacteria antibiotic, has been identified in a variety of fungi such as *Aspergillus* and *Penicillium* spp (*8, 9*).; the same substance is also found in *Monascus* as monascidin A (*10, 11*). Citrinin belongs to the group of polyketides synthesized by the iterative type I polyketide synthase (PKS). Its biosynthesis in *Monascus* originates from a tetraketide arising from the condensation of one acetyl-CoA molecule with three malonyl-CoA molecules (*12*). Although *Monascus* has been widely used in food fermentation, and shown great promise for medicinal development, its application is limited by the nephrotoxic and hepatotoxic properties of citrinin.

The citrinin biosynthetic gene cluster in *Monascus purpureus* BCRC33325 (IFO30873) was proposed in Shimizu's studies (*3*). The polyketide synthase (*pksCT*) and transcriptional activator (*ctnA*) have been proven to be involved in citrinin biosynthesis (*3, 13*). As such, the disruption of *pksCT* results in the phenotype of lost citrinin production (*3*). The *ctnA* gene that encodes the Zn(II)2Cys6 binuclear DNA binding protein is a major activator of citrinin biosynthesis. Consequently, the *ctnA*-disrupted strain of *M. purpureus* also exhibits a significant decrease in citrinin production, to a barely detectable level (*13*). To characterize the citrinin-producing *Monascus* strains, we analyzed the genotype and phenotype of the citrinin synthesized by the various species of *Monascus*. On the basis of the citrinin biosynthetic gene cluster in *M. purpureus* BCRC33325 (IFO30873) (*3, 13*), several primers were designed for polymerase chain reactions (PCR) and Southern hybridizations. Citrinin production was further determined by HPLC analysis.

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Table 1. Strains Used in This Work

strain	species[a]	polyketide synthase (*pksCT*)[b]	activator (*ctnA*)	oxygenase (*orf3*)	source	citrinin production
1. BCRC 31502 (ATCC 16363)	*Monascus pilosus*, type	−	−	−	Japan	−
2. BCRC 38072	*M. pilosus*	−	−	−	Taiwan	−
3. BCRC 31533 (ATCC 16246)	*M. ruber*, type	−	−	−	Nigeria	−
4. BCRC 31523 (ATCC 16378)	*M. ruber*	−	−	−	Taiwan	−
5. BCRC 31534 (ATCC 16366)	*M. ruber*	−	−	−	Switzerland	−
6. BCRC 31535 (ATCC 18199)	*M. ruber*	−	−	−	Canada	−
7. BCRC 33314 (ATCC 16371)	*M. ruber*	−	−	−	Taiwan	−
8. BCRC 33323 (ATCC 18199)	*M. ruber*	−	−	−	Canada	−
9. BCRC 31542 (ATCC 16365)	*M. purpureus*, type	+	+	+	Java	+
10. BCRC 31541 (ATCC 16379)	*M. purpureus*	+	+	+	Taiwan	+
11. BCRC 33325 (IFO 30873)	*M. purpureus*	+	+	+		+
12. BCRC 31615 (DSM 1379)	*M. purpureus*	+	+	+		+
13. BCRC 31506 (CBS 302.78)	*Monascus kaoliang*, type	+	+	+	Taiwan	+
14. BCRC 33446 (ATCC 200613)	*Monascus sanguineus*, type	+	−	−	Iraq	−
15. BCRC 33309 (ATCC 16966)	*Monascus barkeri*	−	−	−	Japan	−
16. BCRC 33310 (IMI 282587)	*Monascus floridanus*, type	−	−	−	USA	−
17. BCRC 33640 (ATCC 204397)	*Monascus lunisporas*, type	−	−	−	Japan	−
18. BCRC 33641 (ATCC 200612)	*Monascus pallens*, type	−	−	−	Iraq	−

[a] "Type" indicates type strain. [b] +, positive; −, negative.

Table 2. Primers Used To Amplify Citrinin Related Genes Fragments

primer[a]	20-mer sequence	position[b]
F1	5′-AACGGACAGGAAGAGCGTGC	68−87 (*ctnA*)
F2	5′-ACGAGTGTCAGTTCGGCTCC	483−502 (*ctnA*)
F3	5′-TCGGAAGCGATCATGGACGT	1277−1296 (*ctnA*)
F4	5′-CTCCTTTCCGCGCAATTCCA	760−779 (*ctnA*)
F5	5′-CGTGCACCTCTACAGGGTTC	113−132 (*orf3*)
F6	5′-CTACCAGGCCATGCTGAAGC	447−466 (*orf3*)
F7	5′-GAGTCCCCGAGAAATGGCAT	1445−1464 (*pksCT*)
F8	5′-AACTGGTCTCTTCCCCAAGC	2661−2680 (*pksCT*)
F9	5′-TTAACCGTCTCCTGTCCGGC	4012−4031 (*pksCT*)
F10	5′-TGCCTATCACGTCAACGGCA	5348−5367 (*pksCT*)
F11	5′-ACGTGGACCATGCCGAGAAC	3313−3332 (*pksCT*)
R1	5′-CGTCTGGTGCAGTTAATGCG	958−977 (*ctnA*)
R2	5′-GGTATGGCATCGGTGGTGTG	1568−1587 (*ctnA*)
R3	5′-GAAACGGGGGAGTGGATTGG	700−719 (*orf3*)
R4	5′-GAGGATCGGATGCGGCATTT	1843−1862 (*ctnA*)
R5	5′-TCTTCGATGGCAACCTGGAC	854−873 (*orf3*)
R6	5′-CTGCCATCTTCCAAGCCCAA	239−258 (*orf4*)
R7	5′-AACAACTACGGTGCTTCCGG	2427−2446 (*pksCT*)
R8	5′-CGGGCTCTGGGTACATCAAA	3654−3673 (*pksCT*)
R9	5′-CGGTCTTGAACCTGACGAGG	5090−5109 (*pksCT*)
R10	5′-GAAGGTACTCGGCCAGAAGC	6486−6505 (*pksCT*)
R11	5′-CAATCACATTCCAAGCGGCG	4303−4322 (*pksCT*)
F12	5′-TCGTTATCTAGGCTGGGCCA	678−697 (*ctnA*)
R12	5′-CGCTGTTTGCGATGCAGTAT	2977−2996 (*pksCT*)
R13	5′-GCCGCCCCATTGAAGAATAC	428−447 (*orf3*)

[a] F, forward primer; R, reverse primer. [b] The sites corresponding to those of the *M. purpureus* BCRC33325 (IFO30873) citrinin gene cluster (GenBank accession no. AB243687).

Our results indicated that the distribution of citrinin biosynthetic genes is highly related to the phylogenetic group of *Monascus* spp.

MATERIALS AND METHODS

Strains, Media, and Growth Conditions. Eighteen strains of *Monascus* as listed in **Table 1** were used in this study. All strains were maintained on PDA (DIFCO, Detroit, MI) agar for 1 week, and spore suspensions were obtained by washing the cultured PDA agar plates with distilled water. Mycelia were harvested after incubation for 14 days at 25 °C with constant agitation in liquid medium (7% glycerol, 3% glucose, 3% monosodium glutamate, 1.2% polypetone, 0.2% $NaNO_3$, and 0.1% $MgSO_4 \cdot 7H_2O$).

Genomic DNA Isolation. *Monascus* genomic DNA was extracted according to the method developed by Bingle et al. (*14*). Approximately 0.5 g (squeezed wet weight) of frozen mycelia was ground to a fine power under liquid nitrogen using a mortar and pestle. Proteins were then removed by successive rounds of extraction using phenol and chloroform. Genomic DNA was recovered by precipitation using 2-propanol and then dissolved in TE buffer.

Polymerase Chain Reaction (PCR) and sequencing. The PCR amplification was carried out with a PCR system 2700 thermocycler (Applied Biosystems, Foster City, CA). The 50 μL reaction mixture contained 100 ng of fungal DNA as template, 0.2 mM of primers, 2 units of Taq DNA polymerase, and 800 mM dNTPs. The 11 primer sets listed in **Table 2** were used for the citrinin biosynthetic gene amplification in this study. The reaction conditions included an initial denaturation for 5 min at 96 °C, which was followed by 30 cycles for 1 min at 96 °C, 1 min at 50 °C, and 2 min at 72 °C with a final extension of 10 min at 72 °C. The PCR products were resolved and recovered on 1.2% agarose gels. Cycle sequencing reactions were then carried out using the BigDye, V3.0 kit (Applied Biosystems, Foster City, CA), while DNA sequencing was performed using an ABI Prism 3730 Sequencer (Applied Biosystems, Foster City, CA).

Southern Hybridization. For Southern hybridizations, genomic DNA (10 μg per lane) was digested with the *Hin*dIII restriction enzyme and separated through 1.2% agarose gels by electrophoresis. Southern hybridization analysis was performed using the DIG system (Roche Diagnostics, Mannheim, Germany). The probes of the citrinin biosynthesis genes were labeled through PCR amplification from the genomic DNA of *M. purpureus* BCRC33325 (IFO30873), using the PCR DIG probe synthesis kit (Roche Diagnostics, Mannheim, Germany). The primer sets of citrinin biosynthesis genes were *pksCT*-F8, AACTGGTCTCTTCCCCAAGC; *pksCT*-R12, ATACTGCATCGCAAACAGCG; *ctnA*-F12, TCGTTATCTAGGCTGGGCCA; *ctnA*-R1, CGTCTGGTGCAGTTAATGCG; *orf3*-F5, CGTGCACCTCTACAGGGTTC; and *orf3*-R13, GCCGCCCCATTGAAGAATAC.

Phylogenetic Analysis. Sequence analyses of the amplified DNA were performed using the VectorNTI 9.0 software (InforMax, Frederick, MD). The accession numbers used for the partial β-tubulin genes were as follows: *Monascus* species, DQ299886−DQ299896, AY498587−AY498589, AY498596, AY498598, AY498601, AY498602, and AY498604; *Aspergillus flavus*, M38265; *Aspergillus parasiticus*, L49386; and *Aspergillus fumigatus*, AY048754. The accession numbers used for the polyketide synthase genes were as follows: *Saccharopolyspora erythraea* DEBS (X56107 and X62569), *Aspergillus terreus lovF* (AF141925), *A. terreus lovB* (AF151722), *Penicillium citrinum mlcA*, *mlcB* (AB072893), *Phoma* sp. SQTKS (AY217789), *Cochliobolus heterostrophus pks1* (U68040), *Gibberella moniliformis* FUM1 (AF155773), *M. purpureus pksCT* (AB167465), *Emericella nidulans wA* (X65866), *E. nidulans stcA* (AAC49191), *Aspergillus parasiticus aflC* (AY371490), *Penicillium patulum* 6-MSAS (X55776), *A. parasiticus pksL2* (U52151), and *A. terreus pksM* (U31329). The phylogenetic tree was constructed using the neighbor-joining method (*15*) via the MEGA 3.1 software with 1000 bootstrap replicates.

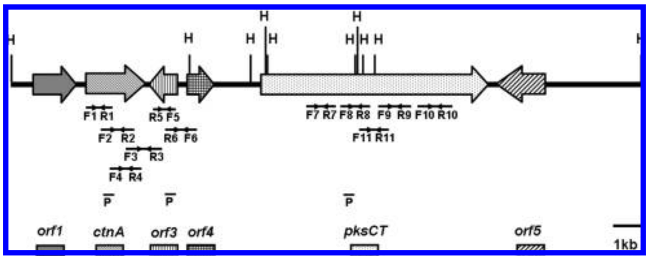

Figure 1. Citrinin gene cluster of *M. purpureus* BCRC33325 (IFO30873) obtained from the GenBank database using accession no. AB243687. The citrinin gene cluster includes dehydrogenase (*orf1*), transcriptional activator (*ctnA*), oxygenase (*orf3*), oxidoreductase (*orf4*), polyketide synthase (*pksCT*), and transporter (*orf5*). The abbreviations F and R indicate the forward and reverse primers, respectively, of *ctnA*, *orf3*, and *pksCT* for the PCR analysis; the abbreviation P indicates the probes for *ctnA*, *orf3*, and *pksCT* in the Southern hybridization analyses.

Measurement of Citrinin. The aliquots of the *M. pilosus* culture were cleared of cells and filtered using a 0.2 mm filter. The supernatants were analyzed using HPLC performed on a Waters system (Waters, Milford, MA) fitted with a μBondapak C_{18} (10 μm) column (Waters, Milford, MA). The HPLC parameters were as follows: solvent gradient with phosphoric acid/acetonitrile/2-propanol 60:35:5 to 25:70:5 (v:v:v) in 10 min; flow rate, 0.8 mL min^{-1}. Fluorescence detection was performed using the 2475 Multi λ Fluorescence (Waters, Milford, MA) set at 330 nm excitation wavelength and 500 nm emission wavelength. A citrinin standard compound (Sigma, St. Louis, MO) was used to confirm the HPLC analysis.

RESULTS

PCR and Southern Hybridization Analysis of Citrinin Biosynthetic Genes. Studies on fungal polyketide biosynthetic genes have indicated that metabolites are largely synthesized by the iterative multifunctional polyketide synthase systems (*16*). Each PKS minimally carries keto-synthase (KS), acyl-transferase (AT), and acyl carrier protein (ACP) domains to catalyze different modifications. To search for the genes related to citrinin biosynthesis, 11 primer sets were designed according to the citrinin gene cluster in *M. purpureus* BCRC33325 (IFO30873) (*3, 13*) (**Table 2** and **Figure 1**). Eighteen strains of the *Monascus* species were used to amplify the genomic DNA of the citrinin biosynthetic genes (**Table 1**). The PCR results showed that the five primer sets of the *pksCT* gene were amplified in *M. purpureus* BCRC31542, BCRC31541, BCRC33325, and BCRC31615, in *M. kaoliang* BCRC31506, and in *M. sanguineus* BCRC33446. In contrast, none of the five primer sets amplified the *pksCT* gene from *M. pilosus*, *M. ruber*, *M. barkeri*, *M. floridanus*, *M. lunisporas*, and *M. pallens*. The PCR analyses of *ctnA* (encoding a transcriptional activator) and *orf3* (encoding oxygenase) with six primer sets were conducted to further verify the citrinin biosynthetic gene cluster. It was found that all strains of *M. purpureus* and *M. kaoliang* possessed both *ctnA* and *orf3* genes, but that these two genes were absent in the *M. sanguineus* strain and other *Monascus* species. Although the PCR condition adopted a low annealing temperature (50 °C), only *M. purpureus* and *M. kaoliang* could yield the PCR products typical of the *pksCT*, *ctnA*, and *orf3* genes, while *M. sanguineus* lacked the PCR products for the *ctnA* and *orf3* genes.

To confirm the citrinin biosynthetic gene distribution in the *Monascus* species, the genomic DNAs were digested by *Hin*dIII for Southern hybridization. The results revealed the presence of *pksCT*, *ctnA*, and *orf3*, which was consistent with the PCR results. Nevertheless, instead of a 3-kb fragment corresponding to the *pksCT* gene in *M. purpureus* and *M. kaoliang*, a 2-kb *Hin*dIII fragment was present in *M. sanguineus*. Meanwhile, the 6.1-kb fragments corresponding to the *ctnA* and *orf3* genes were present in the Southern blot results of *M. purpureus* and *M. kaoliang*, which generally agreed with the size described in the citrinin biosynthetic gene cluster from *M. purpureus* BCRC33325 (IFO30873) (**Figure 2**) (*3*). In addition, the *ctnA* and *orf3* genes were consistently absent in *M. sanguineus* and other *Monascus* species.

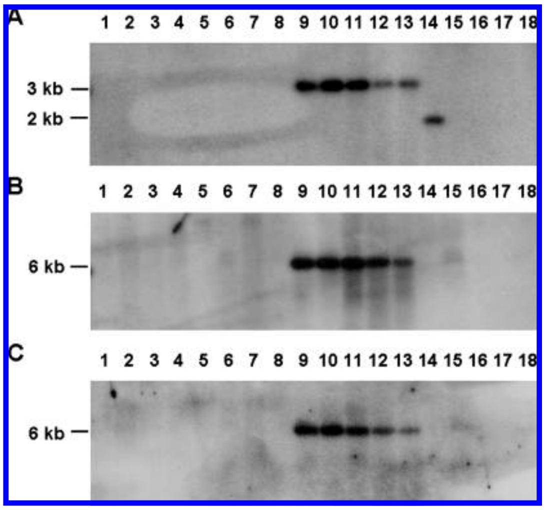

Figure 2. Southern hybridization analyses of the citrinin-related genes. Chromosomal DNA extracted from the *Monascus* species were digested by *Hin*dIII, separated by gel electrophoresis, and hybridized with the (**A**) *pksCT*, (**B**) *ctnA*, and (**C**) *orf3* probes. *Monascus* species: lane 1, *M. pilosus* BCRC31502; lane 2, *M. pilosus* BCRC38072; lane 3, *M. ruber* BCRC31533; lane 4, *M. ruber* BCRC31523; lane 5, *M. ruber* BCRC31534; lane 6, *M. ruber* BCRC31535; lane 7, *M. ruber* BCRC33314; lane 8, *M. ruber* BCRC33323; lane 9, *M. purpureus* BCRC31542; lane 10, *M. purpureus* BCRC31541; lane 11, *M. purpureus* BCRC33325; lane 12, *M. purpureus* BCRC31615; lane 13, *M. kaoliang* BCRC31506; lane 14, *M. sanguineus* BCRC33446; lane 15, *M. barkeri* BCRC33309; lane 16, *M. floridanus* BCRC33310; lane 17, *M. lunisporas* BCRC33640; and lane 18, *M. pallens* BCRC33641.

Sequence and Phylogenetic Analysis of *pksCT*, *ctnA*, *orf3*, and β-Tubulin Genes. The candidate PCR products of *pksCT*, *ctnA*, and *orf3* were further sequenced and analyzed. The result of the 1-kb amplified DNA showed that the AT domain of *pksCT* shared a high similarity among *M. purpureus* BCRC31542, BCRC31541, BCRC33325, and BCRC31615; *M. kaoliang* BCRC31506; and *M. sanguineus* BCRC33446. Both *M. purpureus* and *M. kaoliang* shared 100% identity, while *M. sanguineus* shared 97% identity with the other species. The sequences from *M. purpureus*, *M. kaoliang*, and *M. sanguineus* all contained the conserved amino acid sequence GHSXG, which was the typical active site of the AT domain. The phylogeny was constructed according to the conserved AT domain (**Figure 3**). The *pksCT* from *M. purpureus*, *M. kaoliang*, and *M. sanguineus* belonged to the nonreduced polyketide structural type. *M. sanguineus* was placed in a branch that was separate from *M. purpureus* and *M. kaoliang*. The KS domain of *pksCT* was further sequenced. The sequences from *M. purpureus* and *M. kaoliang* contained the conserved amino acid sequence DXACXS, which was the typical active site of the KS domain. However, the sequence revealed a gap in the upstream of the KS domain of *M. sanguineus* (**Figure 4**); the out-of-frame *pksCT* may result in a nonfunctional polyketide synthase. The active site of the KS domain, DXPCXS, was also different from the typical conserved sequence. Obviously, this *pksCT* was divergent with *M. purpureus* and *M. kaoliang*.

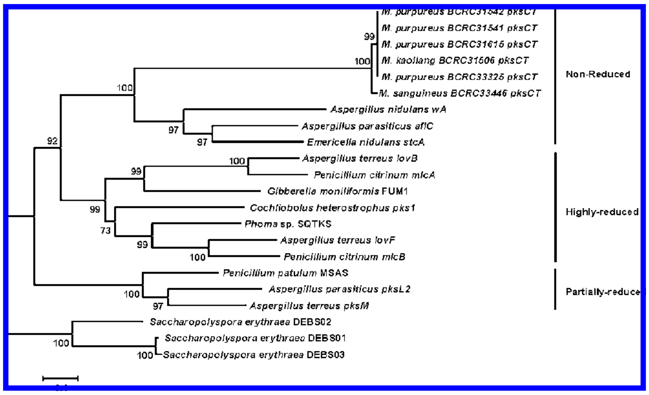

Figure 3. Phylogenetic tree of PKSs from *Monascus* and various organisms. The phylogeny of PKSs based on the conserved acyl-transferase domain with 300 amino acids was constructed and rooted using the acyl-transferase domains of *S. erythraea* DEBS (X56107 and X62569). The accession numbers for the polyketide synthase genes were as follows: *A. terreus lovF* (AF141925), *A. terreus lovB* (AF151722), *P. citrinum mlcA*, *mlcB* (AB072893), *Phoma* sp. SQTKS (AY217789), *C. heterostrophus pks1* (U68040), *G. moniliformis* FUM1 (AF155773), *M. purpureus pksCT* (AB167465), *E. nidulans wA* (X65866), *E. nidulans stcA* (AAC49191), *A. parasiticus aflC* (AY371490), *P. patulum* 6-MSAS (X55776), *A. parasiticus pksL2* (U52151), and *A. terreus pksM* (U31329). Bootstrap values are shown in the nodes according to the 1000 replications. Only bootstrap values >50% are shown.

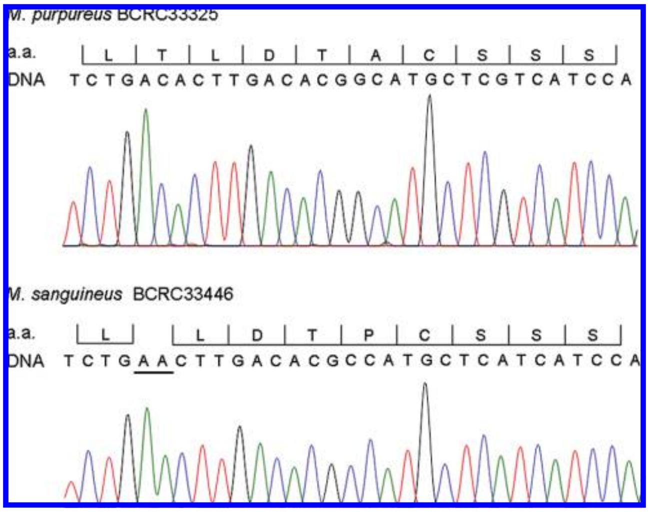

Figure 4. Sequence analyses of the *pksCT* KS domain. The deduced conserved amino acid sequence of the typical active site of the KS domain, DXACXS, was found in *M. purpureus* BCRC33325. A gap, CTGA-ACTT, was observed in the upstream of the *pksCT* KS domain of *M. sanguineus* BCRC33446. The sequencing of *M. sanguineus* BCRC33446 was carried out in triplicate.

The amplified DNA results also showed that the *ctnA* gene shared 100% similarity among *M. purpureus* BCRC31542, BCRC31541, BCRC33325, and BCRC31615, as well as *M. kaoliang* BCRC31506. The *ctnA* gene-encoded transcription factor has been suggested to be a positive regulatory protein involved in citrinin biosynthesis in *M. purpureus* BCRC33325 (IFO30873) (*13*). The arrangement of the cysteine-rich nucleotide-binding domain indicated that the consensus sequence $CX_2CX_6CX_6CX_2CX_6C$ represented a Zn(II)2Cys6-type zinc finger (*13*).

A phylogenetic characterization using the partial β-tubulin gene as a molecular differentiation marker was likewise conducted to further explore the evolutionary history of the various *Monascus* species. The result of the phylogenetic analysis of the partial β-tubulin gene placed both *M. purpureus* and *M. kaoliang* in the same clade (**Figure 5**). On the other hand, *M. ruber*, *M. pilosus*, and *M. barkeri* were placed in the same clade. In addition, *M. sanguineus* was placed in a branch separate from *M. purpureus* and *M. kaoliang*.

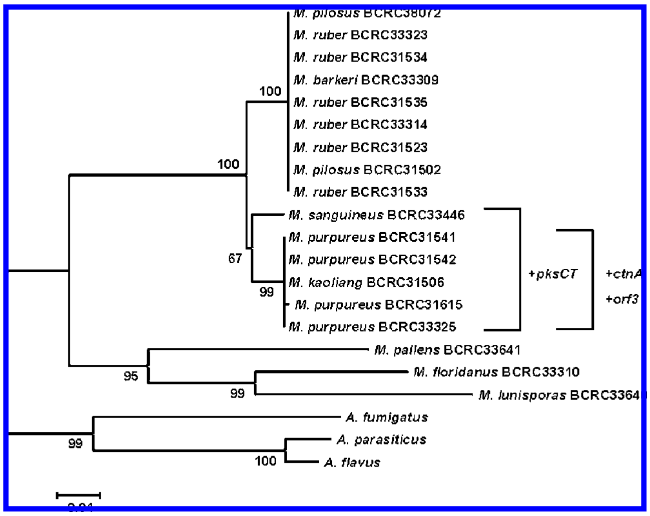

Figure 5. Phylogeny of the *Monascus* species based on the partial β-tubulin gene. The partial β-tubulin genes of the *Monascus* species with the following accession numbers were used: DQ299886−DQ299896, AY498587−AY498589, AY498596, AY498598, AY498601, AY498602, and AY498604. The accession numbers of the β-tubulin genes were used as outgroups of the following: *A. flavus* (M38265), *A. parasiticus* (L49386), and *A. fumigatus* (AY048754). Bootstrap values are shown in the nodes according to 1000 replications. Only bootstrap values >50% are shown.

Analysis of Citrinin Production. In previous studies, the citrinin production in *M. purpureus* BCRC33325 (IFO30873) was shown to progress with the duration of cultivation (*3, 13*). In this study, the amounts of citrinin produced from *Monascus* spp. were determined by HPLC after 14 days cultivation. The results indicated that citrinin was only produced in *M. purpureus* BCRC31542, BCRC31541, BCRC33325, and BCRC31615 and *M. kaoliang* BCRC31506 (**Table 3**). In contrast, the other *Monascus* species did not produce any citrinin.

DISCUSSION

Citrinin is a known hepato-nephrotoxin found in the *Aspergillus*, *Penicillium*, and *Monascus* species, and has been identified as a contaminant in several foods (*8, 9*). Citrinin accumulation in the mitochondria induces apoptosis at the cellular level (*17, 18*). *Monascus*-related products have been used in herbal medicine because their monacolin K content shows the capability to inhibit cholesterol synthesis (*1*). To avoid the negative effects of citrinin, it is important to identify the non-citrinin-producing *Monascus* strains or to eliminate the production of citrinin in *Monascus*. Recently, the citrinin biosynthesis gene cluster has revealed that citrinin is synthesized by polyketide synthase in *M. purpureus* BCRC33325 (IFO30873) (*3*). Moreover, the transcription factor Zn(II)2Cys6 binuclear DNA binding protein is also involved in the regulation of citrinin biosynthesis (*13*). In this study, the distribution of the citrinin biosynthesis genes (**Figure 1**) and the production of citrinin were examined in various *Monascus* species.

Interestingly, the PCR and Southern hybridization results demonstrated that only *M. purpureus*, *M. kaoliang*, and *M. sanguineus* contained the *pksCT* gene encoding polyketide

Table 3. Concentration of Citrinin Produced by *Monascus* Species[a]

	M. purpureus				*M. kaoliang*:
	BCRC31542	BCRC31541	BCRC33325	BCRC31615	BCRC31506
citrinin (mg/g mycelia)[b]	0.33 ± 0.14	15.35 ± 6.17	6.23 ± 1.74	6.56 ± 2.82	2.71 ± 0.28

[a] *Monascus* species were harvested after the cultivation of 14 days. [b] Citrinin was detected by HPLC with the fluorescence detector under the culture condition described in Materials and Methods.

synthase of citrinin (**Figure 2**). The sequences of the AT domain of these amplified *pksCT* genes verified that they were highly homologous with the known citrinin gene cluster in *M. purpureus* BCRC33325 (IFO30873) (**Figure 3**). However, a gap was found in the upstream region of the *pksCT* KS domain of *M. sanguineus* BCRC33446. This missing base could cause a frame shift that results in a nonfunctional polyketide synthase (**Figure 4**). In addition, *M. sanguineus* BCRC33446 did not contain the *ctnA* gene encoding Zn(II)2Cys6 binuclear DNA binding protein or the *orf3* gene encoding oxygenase (**Figure 2**). The strain may have had a large deletion of the citrinin biosynthesis gene cluster initiated possibly by a chromosomal breakage, similar to *Aspergillus oryzae* RIB, which has a deletion in the aflatoxin biosynthesis gene cluster (*19*). The sequences of the 5′ *ctnA* gene from *M. purpureus* and *M. kaoliang* were also verified, including the consensus sequence, $CX_2CX_6CX_6CX_2CX_6C$, which represents a Zn(II)2Cys6-type zinc finger. The citrinin-producing phenotype was detected in *M. purpureus* and *M. kaoliang*, whereas neither *M. sanguineus* BCRC33446 nor the other *Monascus* species produced citrinin. These results suggest that the citrinin gene cluster is highly conserved within *M. purpureus* and *M. kaoliang*, while the *pksCT* gene shows high homology in *M. purpureus*, *M. kaoliang*, and *M. sanguineus*. The citrinin production results were consistent with the distribution of the functional citrinin gene cluster, which was only detected in the strains of *M. purpureus* and *M. kaoliang* (**Table 1**).

Blanc et al. had initially proposed that the structure of monascidin A is identical to the citrinin from both the *M. ruber* ATCC96218 and *M. purpureus* CBS109.07 strains (*10*). The citrinin production of *M. ruber* ATCC96218 was further investigated for its biosynthetic pathway, effects of medium-chain fatty acids, and improvement of the red pigment/citrinin production ratio (*12, 20, 21*). Although previous studies have shown that *M. ruber* ATCC96218 can produce citrinin, this strain has unfortunately been misidentified and was corrected to *M. purpureus* (*22, 23*). Park et al. (*22, 23*) have indicated that the D1/D2 regions of the large subunit (LSU) rRNA gene and the β-tubulin gene could be used to examine the phylogenetic relationship between the *Monascus* species. Their findings are in agreement with our result that *M. ruber* and *M. purpureus* can be distinguished into two series (**Figure 5**). Moreover, the *M. ruber* series (*M. ruber*, *M. pilosus*, and *M. barkeri*) and the *M. purpureus* series (*M. purpureus* and *M. kaoliang*) can be grouped by determining the presence or absence of MRT non-LTR retrotransposon in the hybridization pattern, according to the phylogenetic study established with the β-tubulin gene (*24*). Hence, *M. ruber* ATCC96218 is identical to the *M. purpureus* series based on the D1/D2 region of the LSU rRNA gene (*22*). In addition, the strains of *M. pilosus* ATCC62949 and *M. purpureus* BCRC31523 (ATCC16378) were renamed as *M. purpureus* ATCC62949 and *M. ruber* BCRC31523 (ATCC16378), respectively (*22*). Citrinin is only produced in *P. citrinum*, from the examination of *P. citrinum*, *Penicillium corylophilum*, *Penicillium steckii*, *Penicillium sizovae*, and *Penicillium sumatrense* with 79 isolates (*9*). Citrinin has also been reported in *Penicillium expansum* and *Penicillium verrucosum* (*8*). These results show that citrinin is produced in certain species of *Penicillium*, which is quite similar to our findings that only the species of *M. purpureus* and *M. kaoliang* can produce citrinin.

Wang et al. have proposed that citrinin is detectable in each *Monascus* species under a low HPLC flow rate condition (0.15 mL min^{-1}) and when incubated in a YES medium (40 g L^{-1} of yeast extract and 160 g L^{-1} of sucrose) (*25*). However, these results may need to be reexamined, because we did not detect citrinin in some of the species listed in this paper. Moreover, the incubation and analysis conditions in the Wang et al. study may have led to false positive results. It is possible, but unlikely, that another divergent citrinin biosynthesis gene is present in the *M. ruber* series.

In conclusion, the citrinin production in *Monascus* is consistent with the presence of the functional citrinin biosynthesis genes found only in the *M. purpureus* series. Although *M. sanguineus* BCRC 33446 carries the *pksCT* gene, citrinin production was not detected due to a frame shift of *pksCT* and the absence of some other citrinin biosynthesis genes (**Figures 2** and **4**). The *M. ruber*, *M. pilosus*, and *M. barkeri* strains were grouped together on the basis of the classification of the β-tubulin gene. It was found that they did not possess the *pksCT*, *ctnA*, and *orf3* genes and did not produce citrinin. Therefore, citrinin biosynthesis genes and citrinin production can be used for phylogenetic studies and in the exploration of gene evolution for secondary metabolism.

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