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Investigation of monacolin K, yellow pigments, and citrinin production capabilities of *Monascus purpureus* and *Monascus ruber* (*Monascus pilosus*)

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Abstract

Red mold rice (RMR) is a traditional Chinese medicine prepared using *Monascus* fermentation. *Monascus ruber* (*pilosus*) and *Monascus purpureus* have a long history of use as food and medicine. As an economically important starter culture, the relationship between the taxonomy of *Monascus* and production capabilities of secondary metabolites is crucial for the *Monascus* food industry. In this study, monacolin K, monascin, ankaflavin, and citrinin production by *M. purpureus* and *M. ruber* were genomically and chemically investigated. Our findings suggest that *M. purpureus* can produce monascin and ankaflavin in a correlated manner, whereas *M. ruber* produces monascin with minimum ankaflavin. *M. purpureus* is capable of producing citrinin; however, it is unlikely able to produce monacolin K. In contrast, *M. ruber* produces monacolin K, but not citrinin. We suggest that the current monacolin K content-related regulation of *Monascus* food should be revised, and labeling of *Monascus* species should be considered.

Keywords: Ankaflavin, Citrinin, Monacolin K, Monascin, *Monascus purpureus* *Monascus ruber*

1. Introduction

Red mold rice (RMR) is a traditional Chinese medicine prepared using *Monascus* fermentation. Bioactive compounds, including monacolin K (lovastatin) and pigments, are important functional components of RMR. The *Monascus* yellow pigments, in particular, monascin and ankaflavin, are the focus of current *Monascus* functional food research owing to their multiple health benefits [1]. Besides monacolin K and its pigments, the mycotoxin citrinin is also a critical secondary metabolite of *Monascus*.

Among the *Monascus* species, only *Monascus ruber*, *Monascus pilosus*, and *Monascus purpureus* have a long history of food and medicine usage. *M. purpureus* [2] is distinguished from *M. pilosus* and *M. ruber* by colony growth characteristics, pigment production, and molecular genetics [3,4]. *M. pilosus* was first proposed as a novel species by Hawksworth and Pitt in 1983 and has been proven to be synonymous to *M. ruber* [5].

Monacolin K is the best-known bioactive ingredient in RMR. Many current *Monascus* health food regulations are based on the concentrations of monacolin K and citrinin in food [6–8]. Thus, the relationship between the taxonomy of *Monascus* and the monacolin K and citrinin production capabilities of *Monascus* is crucial for the regulation of *Monascus*. However, existing findings remain inconclusive. In this study, monacolin K, azaphilone pigments, and citrinin production by *M. purpureus* and *M. ruber* (*M. pilosus*) was genomically and chemically investigated. Synteny analysis of monacolin K, azaphilone pigment, and citrinin biosynthesis gene clusters was conducted to investigate the biosynthetic capabilities of *M. purpureus* and *M. ruber* (*pilosus*). Forty-eight *Monascus* strains were isolated from 23 traditional Chinese medicine RMR samples, and these isolates along with other 41 *Monascus* strains, were analyzed using high-performance liquid chromatography (HPLC) to explore the relationship between secondary metabolite signatures and *Monascus* species.

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1.1. Hypothesis

The secondary metabolite signatures (including monacolin K, yellow pigment, and citrinin production capability) of *M. purpureus* and *M. ruber* (*M. pilosus*) are species-specific.

2. Materials and methods

2.1. Microorganisms and cultivation

Ninety-five *Monascus* strains, including 26 reference strains [purchased from the Bioresource Collection and Research Center (BCRC, Taiwan) (Table S1 (https://www.jfda-online.com/cgi/editor.cgi?article=3438&window=additional_files&context=journal)), six strains of *M. pilosus*, six strains of *M. purpureus*, and 14 strains of *M. ruber*], and 69 isolated strains (mostly isolated from the Chinese medicine red mold rice, including eight strains of *M. pilosus*, 60 strains of *M. purpureus*, and one strain of *M. ruber*) were used in this study (Table S1 (https://www.jfda-online.com/cgi/editor.cgi?article=3438&window=additional_files&context=journal)). *Monascus* strains were routinely maintained on potato dextrose agar (PDA; HiMedia Laboratories, India).

2.2. DNA extraction, quantification, polymerase chain reaction (PCR), and sequencing

Eight-day-old *Monascus* mycelia cultivated in potato dextrose broth (PDB) medium were used for DNA extraction. Mycelia were ground to a powder in liquid nitrogen and vacuum-dried. *Monascus* mycelium powder (50 mg) was used for DNA extraction using a QIAamp DNA mini kit (QIAGEN GmbH, Germany). DNA concentration was measured using a Qubit™ dsDNA BR assay kit (Life Technologies Corporation, USA) with a Qubit® 2.0 fluorometer (Life Technologies). Partial internal transcribed spacer (ITS)- and β -tubulin-coding genes were amplified and sequenced for phylogenetic analysis [9]. The primer sets used in this study are listed in Table S2 (https://www.jfda-online.com/cgi/editor.cgi?article=3438&window=additional_files&context=journal), and PCR amplifications were performed using EXSEL high-fidelity DNA polymerase (Bersing Ltd., Taiwan).

2.3. Phylogenetic analysis

Geneious R 8.1.6 software (Biomatters Ltd., Auckland, New Zealand) was used for routine sequence management and de novo sequence assembly. MAFFT (ver. 7.017, [10]) plugin of the Geneious

software was used for multiple alignments. MEGA X [11] was used for the maximum-likelihood phylogenetic tree construction (1000 bootstrap replicates). The general time-reversible (GTR; [12]) model was used for all phylogenetic analyses.

2.4. Functional genomic analysis

M. ruber NRRL 1597 (accession No. PRJNA196033) and *M. ruber* FWB13 (accession no. PRJNA433431) and *M. ruber* CBS 127566 (accession no. PRJEB20852), and *M. pilosus* MS-1, YDJ-2, YDJ-1, and K104061 (accession no. PRJNA718072; [13]), *M. purpureus* YY-1 (accession no. PRJNA453427) and *M. purpureus* NRRL 1596 (BCRC 31542^T; accession no. PRJNA204098) whole-genome shotgun assemblies were used for genomic investigations. The *M. pilosus* pigment (strain BCRC 38072; accession no. KC148521.1), and monacolin K (BCRC 38072; accession no. DQ176595.1), and *M. purpureus* citrinin biosynthesis gene clusters (strain BCRC 31542) were used as queries for a BLAST search of the corresponding biosynthesis gene cluster sequences (Table S3 (https://www.jfda-online.com/cgi/editor.cgi?article=3438&window=additional_files&context=journal)). Custom discontinuous BLAST searches were performed using the Geneious R 8.1.6. Mauve alignment [14] and LASTZ 1.02 [15] were used for the synteny analysis of citrinin biosynthesis gene cluster sequences. InterProScan 5.20 was used for protein functional analysis and conserved domain prediction [16].

2.5. HPLC analysis of secondary metabolites

The *Monascus* strains were cultured in PDB medium for metabolite analysis. After eight days of cultivation, mycelia were collected by centrifugation (15 min at 6000×g). HPLC samples were prepared by 95% ethanol extraction of *Monascus* dry mycelium/pellet at 50 °C for one hour and subsequently filtrated through 0.45 μ m PVDF filter. The HPLC analysis method was adopted from a previous report [17]. Isocratic elution (water: acetonitrile: trifluoroacetate = 38:62:0.05 at 1 mL/min) was carried out on a Hitachi D-2000 Elite HPLC system (Hitachi, Japan) with a LUNA C18 column (250 × 4.6 mm, 5 μ m particle size, Phenomenex, USA), fluorescence detector (L-2485 FL, Hitachi), and a photodiode array detector (L2455 DAD detector, Hitachi). The 385 nm absorbance chromatogram (yellow pigment) was used as the metabolite signature. Pure monascin and ankaflavin were provided by SunWay Biotech Co. Ltd. (Taipei, Taiwan).

2.6. Statistical analysis

R 4.1.3, using the vegan package, was used for principal component analysis (PCA) and analysis of similarities (ANOSIM) of secondary metabolite signatures. Other data were visualized and analyzed the Prism 6.01 software (GraphPad Software, USA). $p < 0.05$ was considered as significantly correlated (Pearson's correlation analysis).

3. Results

3.1. *M. purpureus* is predominant *Monascus* species used for traditional Chinese medicine RMR fermentation

The internal transcribed spacer (ITS) region sequence is currently the major molecular marker used for the identification and classification of eukaryotes. β -tubulin gene sequences are also known to be effective molecular taxonomic markers for the classification of fungi [18]. In this study, the taxonomic position of *Monascus* isolates was investigated using a phylogenetic approach based on both ITS and β -tubulin gene sequences. *Penicillium eremophilus* (*Monascus eremophilus*, [5]) was added as an outgroup. Phylogenetic trees based on ITS and β -tubulin sequences showed that most of the *Monascus* strains isolated from RMR belonged to *M. purpureus*, whereas only one isolate (strain DYH) belonged to *M. ruber* (*M. pilosus*). These results indicate that the bioactivity of traditional RMR may largely originate from *M. purpureus* (Figure S1 (https://www.jfda-online.com/cgi/editor.cgi?article=3438&window=additional_files&context=journal)).

3.2. Pigment, monacolin K, and citrinin biosynthesis gene clusters are highly conserved between *M. pilosus* and *M. ruber*

Monascus produces bioactive secondary metabolites including monacolin K, monascin/ankaflavin, and citrinin. However, the ability of the major edible *Monascus* species, *M. purpureus*, *M. pilosus*, and *M. ruber* to produce these specific secondary metabolites is inconclusive. In this study, synteny analysis of the biosynthesis gene cluster of monacolin K, monascin/ankaflavin, and citrinin was performed to investigate the production capabilities of major secondary metabolites and taxonomic relationships among *M. purpureus*, *M. pilosus*, and *M. ruber*. The sequences of pigment biosynthetic gene clusters were highly conserved (>99.9%) between *M. ruber* and *M. pilosus* genomes. In contrast, the pigment

biosynthesis gene cluster in the *M. purpureus* genome showed less homology to its counterparts in *M. ruber* and *M. pilosus* (Fig. 1(A)). A conserved region of the pigment biosynthesis gene cluster in *M. ruber* and *M. pilosus* strains was confirmed using PCR with the primer set RubPil F/R (Table S2 (https://www.jfda-online.com/cgi/editor.cgi?article=3438&window=additional_files&context=journal)) in all *Monascus* strains. Despite minor differences in gene cluster structure, all *M. purpureus*, *M. pilosus*, and *M. ruber* species are capable of producing yellow, orange, and red pigments.

The monacolin K (MK) biosynthetic gene cluster was also highly conserved (95.0–99.7%) between *M. ruber* and *M. pilosus*. However, all the *M. purpureus* genomes contained only two fragments of the MK gene cluster. One of the residual MK biosynthesis gene clusters contains only a truncated *mokH* gene (Δ *mokH*), and another residual fragment of the MK gene cluster contains recombined and truncated *mokA*, *mokB*, and *mokC* genes. PCR analysis showed that these two residual gene cluster fragments were highly conserved among *M. purpureus* strains. Sequence analysis of Δ *mokH* showed that the Zn (2)-Cys (6) fungal-type transcription factor motif of the *mokH* protein, which is a key activator of MK biosynthesis, was lost. The presence of Δ *mokH* in all *M. purpureus* strains was confirmed using PCR with the event-specific primer set MPus F/MPus R (Fig. 1(B)). The residual MK gene cluster fragments located on different chromosomes also indicated that a large-scale recombination event occurred in the last common ancestor (LCA) of all *M. purpureus* strains (Fig. 1(B)) and thus, *M. purpureus* was unable to produce monacolin K.

The citrinin biosynthesis gene cluster was firstly cloned from *M. purpureus* [19] (accession no. EU309474.1). *M. purpureus* strains shared highly conserved citrinin biosynthesis gene clusters (>99.9% among strains NRRL1596, YY-1, and GenBank EU309474.1). However, only two parts of the citrinin biosynthesis gene cluster were present in both *M. pilosus* MS-1 and *M. ruber* NRRL 1597 genomes (Fig. 1(C)). One residual citrinin biosynthesis gene sequence contained *ctnF* and *ctnR1*, and the other contained *ctnH*, *ctnI*, and a truncated *ctnG* gene. The loss of the major citrinin synthesis gene, *pksCT*, indicates that *M. pilosus* and *M. ruber* are not capable of producing citrinin. Remarkably, the residual citrinin gene sequences were nearly identical between *M. pilosus* MS-1 and *M. ruber* NRRL 1597. These two regions of citrinin gene sequence also highly conserved (92.69–96.30% nucleotide similarity) among *M. purpureus*, *M. pilosus*, and *M. ruber*.

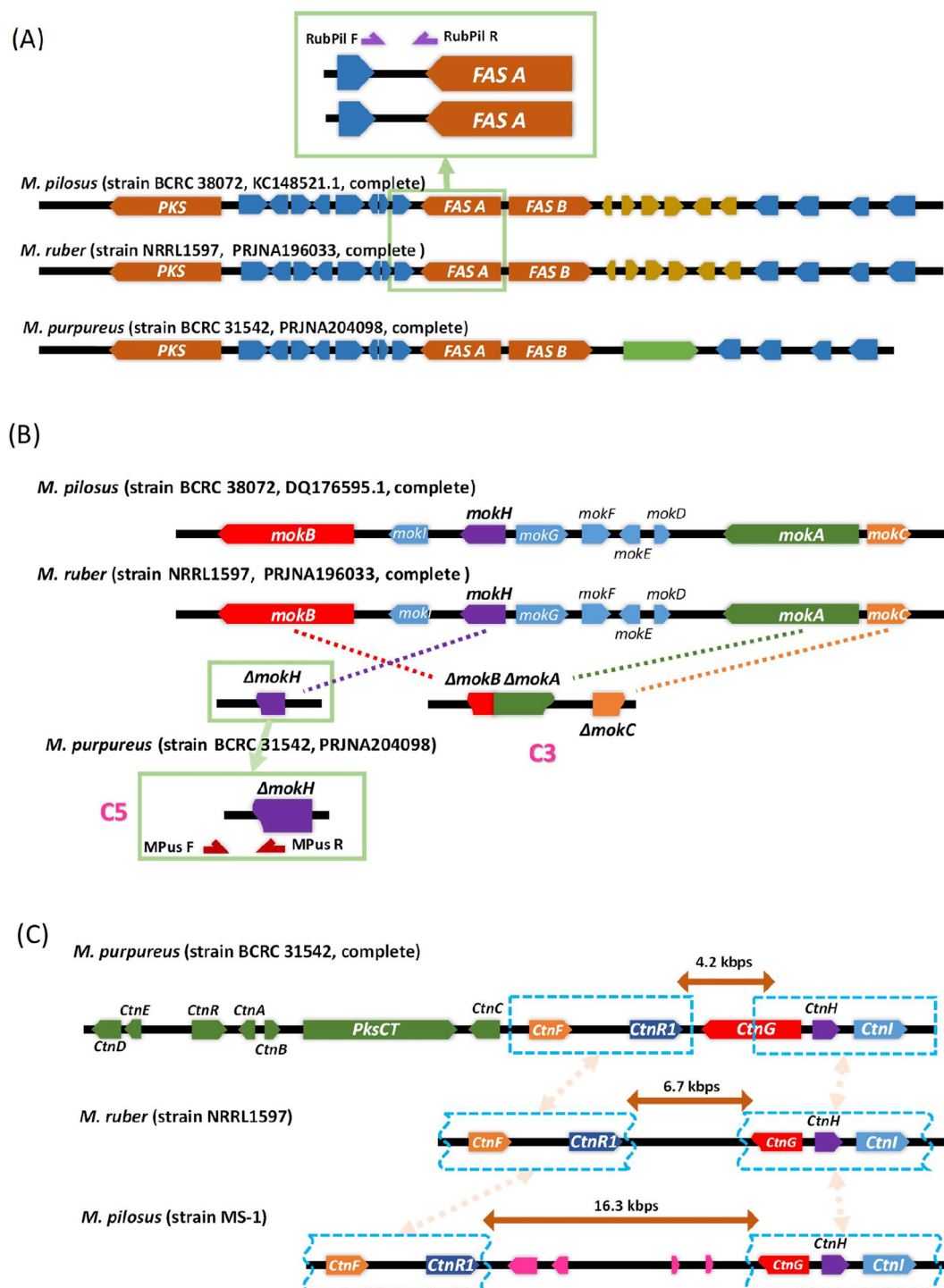


Fig. 1. The pigment, monacolin K, and citrinin biosynthesis gene clusters in *M. pilosus*, *M. ruber* NRRL 1597 and *M. purpureus* BCRC 31542 genomes. These gene clusters were nearly identical between *M. pilosus* and *M. ruber*. (A) Pigment biosynthesis gene clusters. Functional pigment biosynthesis gene clusters were present in all three species. *M. pilosus* (*M. ruber*)-specific primer set RubPil is targeted to the upstream sequences of FASA gene. (B) Monacolin K biosynthesis gene clusters. Besides lost most part of monacolin K biosynthesis gene cluster sequences, the residual two sequence fragments located at two different chromosomes in *M. purpureus* (C3 and C5 of *M. purpureus* YY-1 genome). (C) Citrinin biosynthesis gene clusters. Two types of residual citrinin biosynthesis gene cluster present in the *M. pilosus* and *M. ruber* genomes.

However, the insert sequences between the two residual citrinin gene cluster sequences are different in *M. pilosus* MS-1 (approximately 16.3 kbps,

designated as insert ρ) and *M. ruber* NRRL 1597 (approximately 6.7 kbps, designated as insert π). The insert sequence π contained no ORF, and insert

ρ contained four predicted ORFs with unknown functions. The sequence of insert π shares no homology to insert ρ , and both insert π and ρ have no homology to any other region of the *M. purpureus*, *M. pilosus*, and *M. ruber* genomes (Fig. 2).

3.3. *M. pilosus* and *M. ruber* shared similar signature of yellow pigments production, differing from *M. purpureus*

Ninety-five *Monascus* strains, including 14 strains of *M. pilosus*, 66 strains of *M. purpureus*, 15 strains of *M. ruber*, and 27 RMR samples (Chinese herbal medicine fermented by *M. purpureus*) were used for secondary metabolite analysis. Peak areas of the absorbance spectrum at 385 nm (major absorbance wavelengths of monascin and ankaflavin) were selected for metabolite signature comparison (Fig. 3(A)). PCA analysis of seven peak areas of the absorbance spectrum (385 nm) indicated that the production of citrinin and ankaflavin was closely related to *M. purpureus*, whereas *M. pilosus* was characterized by low-level pigmentation (Fig. 3(B)). Among *M. purpureus*, *M. pilosus*, and *M. ruber*, only *M. purpureus* produced

higher amounts of ankaflavin, which was significantly correlated with monascin in both the mycelium (Pearson's $r = 0.9017$, $p < 0.0001$; Fig. 4(A)) and RMR (Pearson's $r = 0.9393$, $p < 0.0001$; Fig. 4(B)). In contrast, *M. ruber* (*pilosus*) had a higher p10.9 peak area which also significantly correlated with the peak area of monascin (Pearson's $r = 0.9363$, $p < 0.0001$; Fig. 5). ANOSIM analysis also suggested that the metabolite signatures of *M. purpureus* and *M. ruber* (*pilosus*) were significantly different ($R = 0.8517$; $p = 0.001$).

3.4. Average Nucleotide Identity (ANI) and the distribution of insert sequences among *M. pilosus* and *M. ruber* strains suggest that the strains are synonymous

ANI analysis is an alternative method for DNA–DNA hybridization based on genome sequences, and is generally recognized as a standard for taxonomic investigation of microorganisms [20,21]. ANI analysis among *M. purpureus*, *M. pilosus*, and *M. ruber* genomes showed that ANI between *M. purpureus* and *M. ruber* are 94.22–94.30%, while ANI

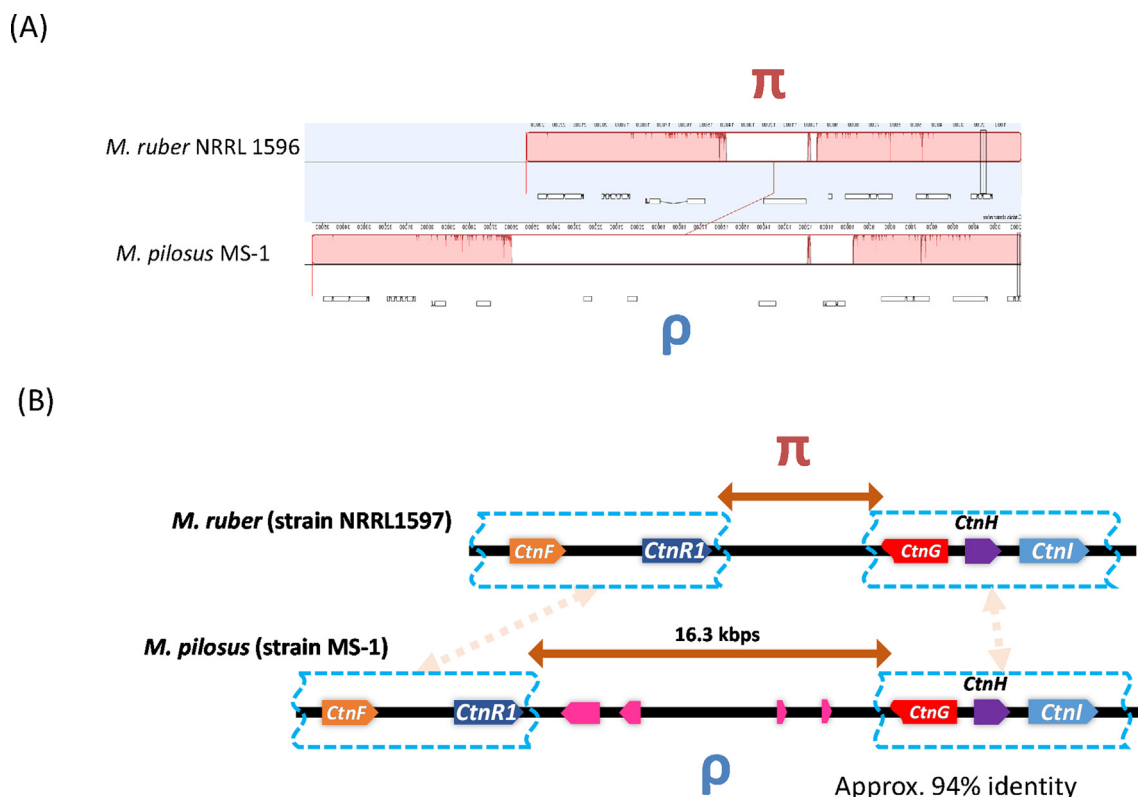


Fig. 2. Residual citrinin biosynthesis gene clusters in *M. pilosus* (strain MS-1) and *M. ruber* (strain NRRL 1597) genomes. (A) Mauve alignment analysis revealed two types of insert sequences flanked by highly conserved residual citrinin biosynthesis gene cluster sequences. π : The insert sequence region of citrinin biosynthesis gene cluster in *M. ruber* NRRL 1597. ρ : The insert sequence region of citrinin biosynthesis gene cluster in *M. pilosus* MS-1. Shaded region in red: sequence similarity of conserved region. Blank: regions with no homology. (B) Schematic diagram of residual citrinin biosynthesis gene clusters. Core citrinin biosynthesis gene *pksCT* was not found in both genomes.

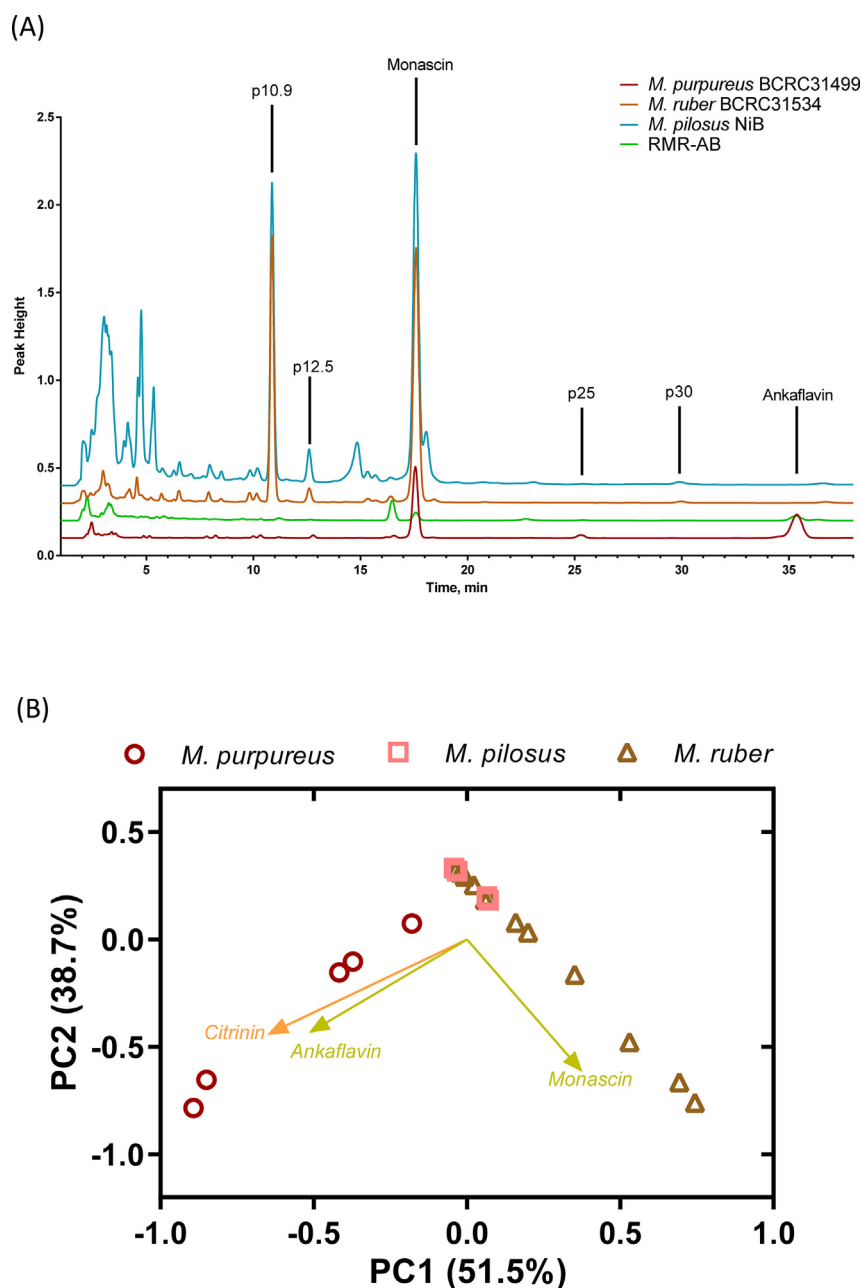


Fig. 3. *M. pilosus* and *M. ruber* shared common HPLC absorbance chromatogram signature at 385 nm that was different from that of *M. purpureus*. (A) Comparison of absorbance chromatograms (385 nm) and selected peaks. p10.9: Unidentified peak with 10.9 min retention time. pMS: peak of monascin. pAK: peak of ankaflavin. RMR-AB: Red mold rice AB which purchased from a Chinese herbal medicine pharmacy (prepared with *M. purpureus*). (B) PCA analysis of 385 nm absorbance chromatogram (peak area of seven peaks including citrinin, monascin, ankaflavin, p10.9, p12.5, p25, and p30). Citrinin and ankaflavin production were correlated to *M. purpureus* and differentiate it from *M. ruber* (*pilosus*).

between *M. purpureus* and *M. pilosus* are 94.20–94.29%. These interspecies ANI were significantly lower than intraspecies ANI (*M. purpureus* to *M. purpureus*, 99.89%; *M. pilosus* to *M. pilosus*, 99.92–99.98%; *M. ruber* to *M. ruber*, 99.86–99.89%; Figure S2 (https://www.jfda-online.com/cgi/editor.cgi?article=3438&window=additional_files&context=journal)). However, the interspecies ANI between

M. pilosus and *M. ruber* are 99.87–99.98%, which is not significantly lower than intraspecies ANI.

The insert sequences π (in the *M. ruber* NRRL 1596 genome) and ρ (in the *M. pilosus* MS-1 genome) of *M. ruber* and *M. pilosus* strains were further investigated by partial PCR amplification and sequencing (Fig. 6 (A)). Although the DNA sequences of π and ρ inserts are highly conserved (89.9–99.99% mean

pairwise identity) among *Monascus* strains, only one type (π or ρ) of insert sequence exists in *Monascus* genome and not correlate with specific *Monascus* species (Fig. 6 (B)). *M. pilosus* K104061, MS-1, YDJ-1, and *M. ruber* FWB-13 genomes had ρ type insert sequences (99.7%, 99.7%, 99.7%, and 99.9% identical sites, respectively). *M. pilosus* YDJ-2 and *M. ruber* CBS 127566 genomes have a π -type insert sequence (with 99.8% and 99.4% identical sites, respectively; Fig. 6 (C) and (D)).

4. Discussion

M. ruber, *M. pilosus*, and *M. purpureus* have a long history of food and medicinal usage. As an economically important starter culture, the relationship between the taxonomy of *Monascus* and the production capabilities of secondary metabolites is crucial for food and regulation in *Monascus*.

M. pilosus and *M. ruber* have been proposed to be synonymous in several previous studies [5,9]. Barbosa et al. [5] also reported that pigmentation is not

a robust taxonomic key for distinguishing *M. pilosus* from *M. ruber*. In our study, synteny analysis showed that *M. pilosus* and *M. ruber* share highly conserved and complete monacolin K and azaphilone pigment biosynthesis gene clusters, which were significantly different from those of *M. purpureus* (Fig. 1 and Fig. 6). Although the pigment synthesis gene cluster in the *M. purpureus* genome is complete, all *M. purpureus* strains have only two highly conserved residual monacolin K biosynthesis gene cluster sequences in which all residual genes appeared to be nonfunctional (Fig. 1(B)). Kwon et al. [22] reported that most part of monacolin K biosynthesis gene clusters were lost in *M. purpureus* NRRL 1596 and *M. purpureus* YY-1. Our findings suggest that all *M. purpureus* strains shared a highly conserved residual monacolin K biosynthesis gene cluster with the disrupted *mokH* gene (confirmed

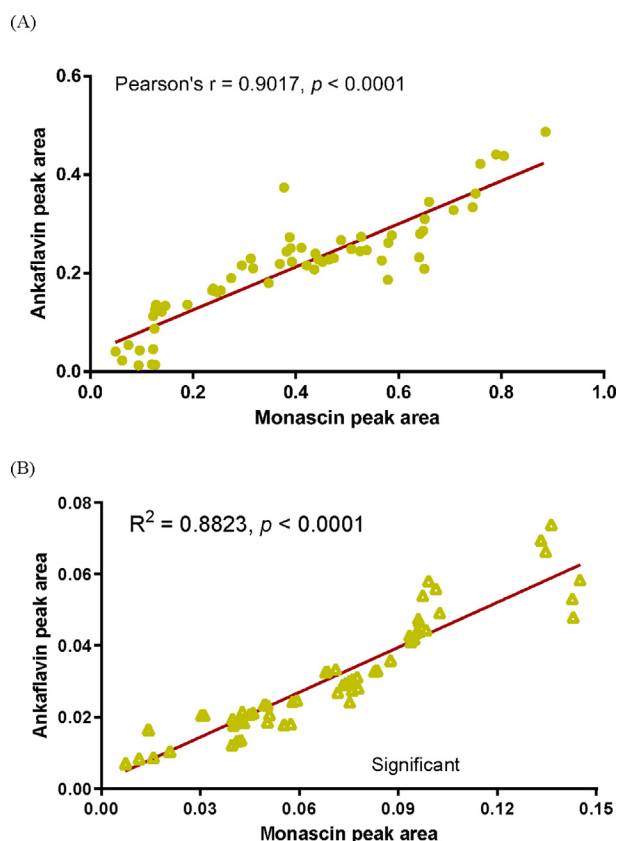


Fig. 4. Monascin and ankaflavin production are significantly correlated in *M. purpureus*. (A) Monascin and ankaflavin production are significantly correlated in *M. purpureus* (20 strains, triplicated test) cultivated in PDB medium. (B) Monascin and ankaflavin production were also significantly correlated in RMRs (23 samples).

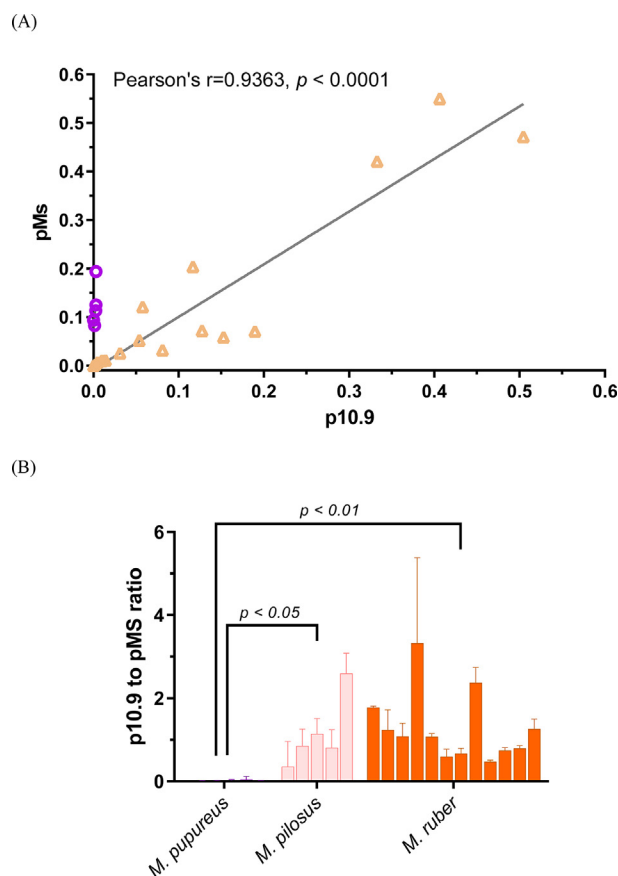


Fig. 5. Besides monascin and ankaflavin production, absorbance chromatogram (385 nm) also revealed specific metabolite signature of *M. ruber* (*pilosus*). (A) *M. pilosus* (*ruber*) and *M. purpureus* could be differentiated with the ratio of peak area of monascin (pMS) to p10.9. Purple circle: *M. purpureus* strains. Pink triangle: *M. pilosus/ruber* strains. Pearson's r for the correlation analysis between peak area of pMS and p10.9 in *M. pilosus/ruber* strains. (B) Peak area ratios of p10.9 to pMS were significantly different between *M. purpureus* and *M. ruber* (*pilosus*).

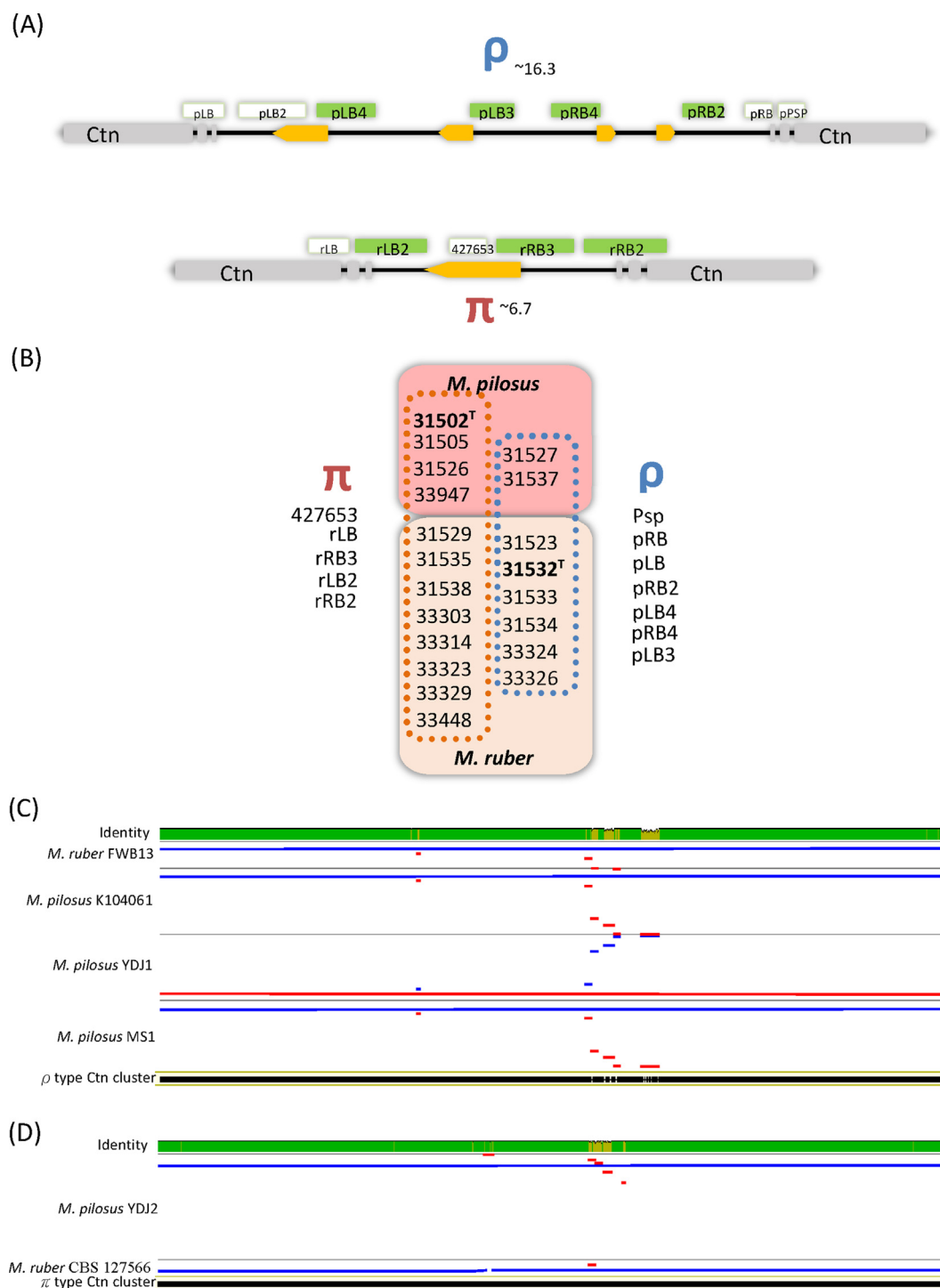


Fig. 6. The distribution of π and ρ insert sequences in *M. pilosus* and *M. ruber* strains. (A) Primer sets designed for π and ρ insert sequences PCR and sequencing. Yellow arrow: predicted genes in insert sequences. White box: regions for PCR amplification. Green box: PCR and sequenced region. (B) The distribution of π and ρ insert sequences in *M. pilosus* and *M. ruber* strains irrelevant to species.

using PCR and sequencing analysis), which indicates that the disruption of the monacolin K biosynthesis gene cluster is species-specific. Chen et al. [23] showed that *mokH* is essential for monacolin K biosynthesis. Higa et al. [24] reported that

M. purpureus NBRC 4478 lacks a complete monacolin K gene sequence, consistent with our findings. The strain-specific disruption of the monacolin K biosynthesis gene cluster suggests that *M. purpureus* lost its monacolin K-producing capability.

Synteny analysis also revealed that only *M. purpureus* had a complete citrinin biosynthesis gene cluster. *M. pilosus* and *M. ruber* shared highly conserved residual gene cluster sequences in which major citrinin biosynthesis genes, such as *pksCT*, were lost (Fig. 6 (A), confirmed using PCR and sequencing analysis). Chen et al. [25] showed that, among *Monascus* species, *pksCT* was only present in *M. purpureus* and *Monascus sanguineus*, consistent with our findings. Shimizu et al. [26] reported that disruption of the *pksCT* gene abolished citrinin production. Higa et al. [24] also reported that *M. pilosus* NBRC 4520 and *M. ruber* NBRC 4483 lacked the complete citrinin gene sequence, consistent with our findings. Since all strains of *M. ruber (pilosus)* share two types of residual citrinin biosynthesis gene clusters and all *pksCT* genes were missing, we suggest that *M. ruber (pilosus)* is unable to produce citrinin.

Monacolin K, citrinin, and red pigments have been reported to possess antimicrobial activities [27]. These antimicrobial secondary metabolites may provide *Monascus* ecological competitiveness at the cost of a metabolic burden. Pigments are also a common UV protection strategy for terrestrial fungi, and may thus be conserved among *M. purpureus*, *M. pilosus*, and *M. ruber*. Loss of monacolin K or citrinin biosynthesis in *M. purpureus* and *M. ruber/M. pilosus* may represent a trade-off between resource competitiveness and metabolic burden during the evolution of these species.

The highly conserved pigment, citrinin, and residual monacolin K biosynthesis gene cluster in *M. purpureus* genomes suggests that major recombination events of secondary metabolite biosynthesis gene clusters occurred during the speciation of *M. purpureus*, indicating that the evolution of these major secondary metabolite gene cluster sequences may correlate with *Monascus* taxonomy. The residual monacolin K and citrinin gene clusters in *M. purpureus* and *M. ruber (pilosus)* also suggest that these three *Monascus* species share a common ancestor capable of producing both monacolin K and citrinin. The production of secondary metabolites is an important adaptation strategy that is usually species-specific fashion [28]. In this study, metabolite analysis revealed that the signature of *M. purpureus* was capable of producing monascin and ankaflavin in a correlated manner (median MS to AK ratio of all *M. purpureus* was 2.016), whereas *M. ruber (pilosus)* produced monascin with minimum ankaflavin. *M. purpureus* is also capable of producing citrinin, but not monacolin K. In contrast, *M. ruber (pilosus)* produces monacolin K, but not citrinin.

M. purpureus, *M. pilosus*, and *M. ruber* were phylogenetically closely related (Figure S1 (https://www.jfda-online.com/cgi/editor.cgi?article=3438&window=additional_files&context=journal)). Our findings indicate that monacolin K, azaphilone pigment, and citrinin biosynthesis gene sequences, as well as production capabilities, are conserved within species of *M. purpureus* and *M. ruber (pilosus)*. However, the monacolin K and citrinin production capabilities of *M. purpureus* and *M. ruber (pilosus)* have been inconclusive in previous studies [29–32]. These controversies may arise from incorrect species identification caused by unreliable morphological taxonomic keys and internal transcribed spacer (ITS) sequence-based phylogenetic analysis. For example, *M. ruber* M7 is a well-known strain with a fully sequenced genome. Our current knowledge of azaphilone pigment biosynthesis and regulation in *Monascus* has been elucidated through comprehensive studies of *M. ruber* M7 [31,33]. Synteny analysis revealed that *M. ruber* M7 has an *M. purpureus*-type pigment [31] and complete citrinin (accession KT781075.1, approximately 42.8 kbps, >99.9% identity to *M. purpureus* YY-1 with 99.55% coverage) biosynthesis gene clusters, which indicate that *M. ruber* M7 may belong to *M. purpureus*.

Monacolin K and citrinin contents in *Monascus* products are legally regulated in many countries. The U.S. Food and Drug Administration (FDA) has determined that red yeast rice products containing more than trace amounts of monacolin K are unapproved new drugs and cannot be sold legally as dietary supplements [34]. Citrinin is particularly crucial for *Monascus* products because it is a major safety concern for *Monascus*. Our investigation helps to clarify the production capabilities of important secondary metabolites and their relation to *Monascus* species, which will augment *Monascus* product regulation, such as labeling or approval of *Monascus* species used for production.

In conclusion, our findings suggest that *M. purpureus* can produce monascin and ankaflavin in a correlated fashion, whereas *M. ruber (pilosus)* produces monascin with minimum ankaflavin. *M. purpureus* is also capable of producing citrinin, but not monacolin K. In contrast, *M. ruber (pilosus)* produces monacolin K, but not citrinin. These results confirm our hypothesis that the secondary metabolite signatures of *M. purpureus* and *M. ruber (pilosus)* are species-specific. Since *M. purpureus* is the predominant species used for traditional RMR preparation, the current monacolin K (lovastatin) content-related regulation of *Monascus* food should be revised. The common bioactive *Monascus* metabolite monascin is

a good alternative functional component for *Monascus* product regulation, and labeling of *Monascus* species should be considered.

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