Doctoral Dissertation

Integrative analysis of genome and secondary metabolites in *Monascus* species.

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Abstract

Fermented foods made by fermenting substrates with microorganisms are eaten all over the world. Manufacturing control is complicated because microorganisms ferment. The final product will change depending on the culture conditions such as temperature, moisture content, culture days, and additives. Therefore, it is very important in the study of fermented foods to reveal the final product that humans eat, that is, the phenotype. The purpose of this study was to clarify the phenotype from genome and metabolite analysis using *Monascus*, which is an economically important fermented food. *Monascus* is used in cooking in East Asian countries such as China, Japan and South Korea. In industry, three types of *Monascus*, *M. pilosus*, *M. purpureus*, and *M. ruber* are used for food fermentation. However, there are lacks of information on the genomes and secondary metabolites of these strains. Here, we report the genomic analysis and secondary metabolites produced by the NBRC standard strains *M. pilosus* NBRC4520, *M. purpureus* NBRC4478, and *M. ruber* NBRC4483. We examined the diversity of secondary metabolites in the three *Monascus* species, both at the metabolite level and at the genomic level by LCMS analysis. Illumina MiSeq 300 bp pair-end sequencing produced 17 million high-quality short reads of various types, equivalent to 200 times the genome size. LCMS analysis was used to measure pigments and their associated metabolites. The colors of the liquid media corresponding to the pigments produced by the three species and their associated metabolites were very different from each other. The gene clusters for secondary metabolite biosynthesis of the three *Monascus* species are also divergent, confirming that *M.pilosus* and *M.purpureus* are chemically taxonomically different. A comparison of the secondary metabolites produced also revealed differences between the three species. In particular, *M.pilosus* NBRC4520 and *M.ruber* NBRC4483 had no conserved genes that make mycotoxins citrinin and no metabolites detected. Therefore, *M.pilosus* NBRC4520 and *M.ruber* NBRC4483 are considered to be the safest strains of *Monascus*. Our findings are important for

improving the use of *Monascus* species in the food industry. *Monascus* makes important metabolites such as statins, azaphilone pigments, and citrinin that have an effect on human health. It is important to clarify the phenotype of microorganisms in terms of genome and metabolites for human consumption. In this study, we compared three species of *Monascus* from the genome and metabolites, and confirmed *Monascus*, which has high safety and health effects as a phenotype. It is important to integrate the genome and metabolites to identify the phenotype that humans eat. Analyzing the phenotype can be applied not only to red yeast rice but also to other fermented foods such as natto, yogurt, and tempeh. The fermentation process of microorganisms is very complicated. By integrating genome analysis and metabolome analysis and standardizing phenotypic analysis, fermented foods can contribute to our health more scientifically.

Keywords: *Monascus*, bioinformatics, genome analysis, metabolomics analysis

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Chapter1: Introduction

1.1 *Monascus*

Monascus is a filamentous fungus that produces a red pigment. Isolated from red yeast rice by 1884 Van Tieghem. [1] Van Tieghem named *Monascus ruber*. Based on the classification of Ainsworth and Bisby, the genus *Monascus* has been determined to belong to the family *Monoscaceae*, the order *Eurotiales*, the subclass *Eurotiomycetidae*, and the class *Eurotiomycetes*. Many *Monascus* species have been identified so far, including *M. pilosus*, *M. ruber*, *M. purpureus*, *M. floridanus*, *M. lunisporas*, and *M. mellicola* (Table 1) [2]. Red yeast rice is introduced in the "Compendium of Materia Medica" compiled in China in 1578. In the olden days, it was used as a crude drug as "improves digestion, promotes blood flow, improves spleen, stomach function, and restores energy, repairs trauma, gynecological diseases and postpartum confinement." It is now used worldwide as a food colorant, fermented food and supplement. [3] In Japan, it has been used for tofu-yo, miso, vinegar and sake. [4] From the above, red yeast rice is an indispensable part of our diet. On the other hand, red yeast rice produces a large amount of peculiar secondary metabolites. There are monacolins known as statins [5, 6, 7, 8], azaphilone pigments [9], and citrinin [10]. Each has its own unique bioactivity.

1.2 Monacolin K

Hyperlipidemia is a lifestyle-related disease that is popular among many people around the world. Hyperlipidemia caused by elevated cholesterol increases the risk of death from heart disease. More than 17 million people worldwide die from cardiovascular disease each year. [11] First statin (mevastatin) was discovered in *Penicillium citrinum* by Endo in 1973. [12] However, mevastatin has been confirmed to have side effects in dogs and has not been commercialized. [13] Later, first statin drug, lovastatin (monacolin K), was discovered. Endo discovered monacolin K in 1979 in the culture medium of *Monascus ruber* (Fig. 1). [5] Since then, numerous monacolin analogs have been reported, including monacolin J, monacolin L [7], and monacolin X [6] (Fig. 2) . Statins are widely used worldwide and are still used as first-line drugs. Monacolin K inhibits hydroxyl methyl

glutaryl-coenzyme A (HMG-CoA) reductase, an enzyme in the mevalonate pathway in the liver (Fig. 3) [11]. Inhibiting HMG-CoA reductase reduces cholesterol synthesis. Inhibition of cholesterol synthesis in the liver results in suppression of the production of very low-density lipoprotein (VLDL) in the liver. By suppressing the production of VLDL, the concentration in blood decreases. As a result, low-density lipoprotein (LDL) cholesterol in the blood is lowered, hyperlipidemia is improved, and arteriosclerosis is prevented [14]. Lovastatin is also expected to improve osteoporosis [15] and prevent Alzheimer's disease. [16] Therefore, it is considered that foods and supplements containing lovastatin (monacolin K) greatly contribute to our health.

1.3 Monascus azaphilone pigment

Azaphilone pigments have an azaphilone skeleton produced by microbial and mushroom polyketide synthases [9, 17]. The azaphilone pigment produced by red yeast rice is called *Monascus* azaphilone pigment (MonAzP). MonAzP has been used as a natural colorant for over 2000 years. Today, food has an important role to play. MonAzP is an ingredient in sausages; fermented tofu (Tofu-yo), red rice wine, kamaboko, and various others preserved dried meat and fish products in Asia. In Europe, it is used as an alternative to the preservation of meat products such as sausages and ham and the coloring with nitrates and nitrites. [18] MonAzP is mainly known as the yellow pigments Monascin and ankaflvin, the orange pigments rubropunctatin and monascorubrin, and the red pigments rubropunctamine and monascorubramine (Figure 4). [9] Since yellow, orange, and red natural pigments are in high demand, research on fermentation production control and production control by genetic recombination is in progress. [19, 20] In recent years, pharmacological studies of MonAzP have been reported. Monascin and ankaflavin have reports on lipid metabolism. It was revealed that monascin and ankaflavin have an agonistic effect on peroxisome proliferator-activated receptor alpha and gamma. [21, 22, 23] Monascin and ankaflavin reduced obesity in obesity models using high fad diet-induced rats and high-fructose diet-induced mice (C57BL6J). [21] Therfore, the yellow pigment is expected to have an anti-obesity effect. Rubropunctamine is also known to have an antioxidant effect. [24] Monascorubrin and monascoribramine have no reports of pharmacological activity. It is hoped that future research will reveal the effects of MonAzP on our health.

1.4 Citrinin

Mycotoxins are natural toxins produced as secondary metabolites by fungi, especially *Aspergillus*, *Penicillium* and *Monascus* species. Citrinin is significant food pollutants [25]. Many plants are used worldwide for medicinal and culinary purposes [26]. Citrinin contamination is of economic and culinary importance that can be easily compromised by mycotoxin contamination. Citrinin is a polyketide mycotoxin produced by several species of the genera *Aspergillus*, *Penicillium*, and *Monascus* [27]. It has since been confirmed that other species of *Penicillium* (*P. expansum* and *P. viridicatum*), and even *Aspergillus* (*A. niveus* and *A. terreus*), produce citrinin [28]. Citrinin is obtained from harvested plant grains under typical storage conditions [29]. Citrinin is primarily known for its toxicity, but there is increasing evidence to support anti-cancer and neuroprotection in particular. Citrinin is primarily known for its toxicity [25], but other biological activities of this mycotoxin, especially anti-cancer and neuroprotection. [30, 31] There is increasing evidence in favor of anti-cancer and neuroprotection. However, it is known to cause renal failure and should be tightly controlled. In fact, it is strictly controlled at 100 ug/kg in Europe EFSA and 2,000 ug/kg in Japan. Quantitative analysis results of citrinin in commercially available red yeast rice supplements have been reported. Surprisingly, citrinin was detected in many food and supplements. [32] 44.24 mg/kg of citrinin was detected in certain supplements. [33] Research on the fermentation control of citrinin is underway, focusing on the fermentation conditions in red yeast rice, but it is not yet complete. [34, 35, 36, 37]

1.5 Genome research of *Monascus*

Next Generation Sequencing (NGS) has made the acquisition of biological genomic information quick and inexpensive. It is easy to obtain a lot of genomic information such as plants, animals, and microorganisms. However, there are few reports of genomic information in red yeast rice. Completed genome decoding of the industrial strain *Monascus purpureus* YY-1 in 2015. [38] Also reported an announcement letter that they obtained whole-genome analysis of *Monascus purpureus* GB-01 in 2019. [39] *Monascus ruber* NRRL 1597 (https://genome.jgi.doe.gov/portal/MonrubNRRL1597_FD/) and

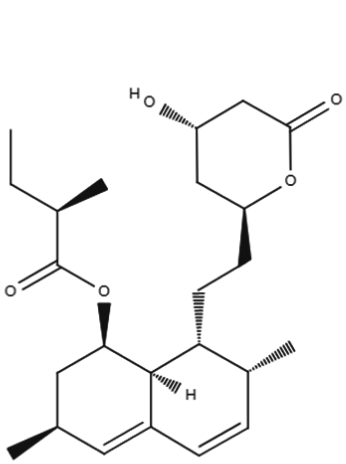
Monascus purpireus NRRL 1596 (https://genome.jgi.doe.gov/portal/MonpurStandDraft_FD/) are genomes on the database. However, the genome data, which is *Monascus ruber* NRRL 1597, don't available. Therefore, in order to deepen the understanding of *Monascus purpureus*, it is necessary to obtain further genomic information and comparative genome analysis.

1.6 Purpurse of this study

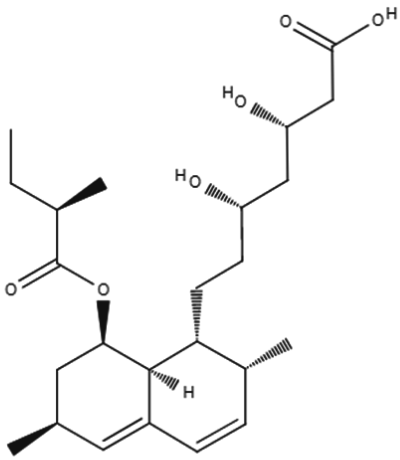
In this study, we determined the genome sequences of *M. pilosus* NBRC4520, *M. purpureus* NBRC4478, and *M.ruber* 4483. The phylogenetic and chemotaxonomic differences between these three species were characterized by analyzing the gene clusters associated with secondary metabolites. These results are considered to give great insight into the safety and effectiveness of red yeast rice, which has been used as a food for many years.

Table 1 List of *Monascus* strains

Species
Monascus pilosus
Monascus purpureus
Monascus ruber
Monascus floridanus
Monascus lunisporas
Monascus mellicola
Monascus pallens
Monascus argentinensi
Monascus eremophilus
Monascus flavipigmentosus
Monascus recifensis
Monascus sanguineus

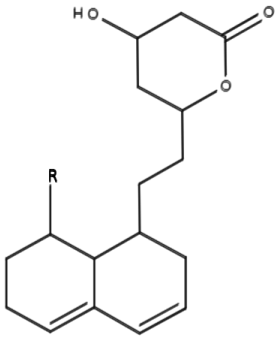


Monacolin K Lacton form



Monacolin K hydroxyl acid

Figure 1 Chemical structure of Monacolin K lacton and acid form.



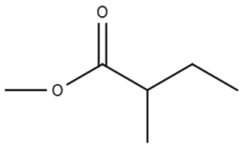
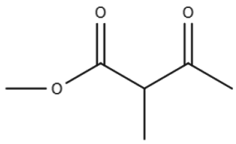
R	Compound
OH	Monacolin J
	Monacolin K
H	Monacolin L
	Monacolin X

Figure 2 Chemical structure of monacolins analog.

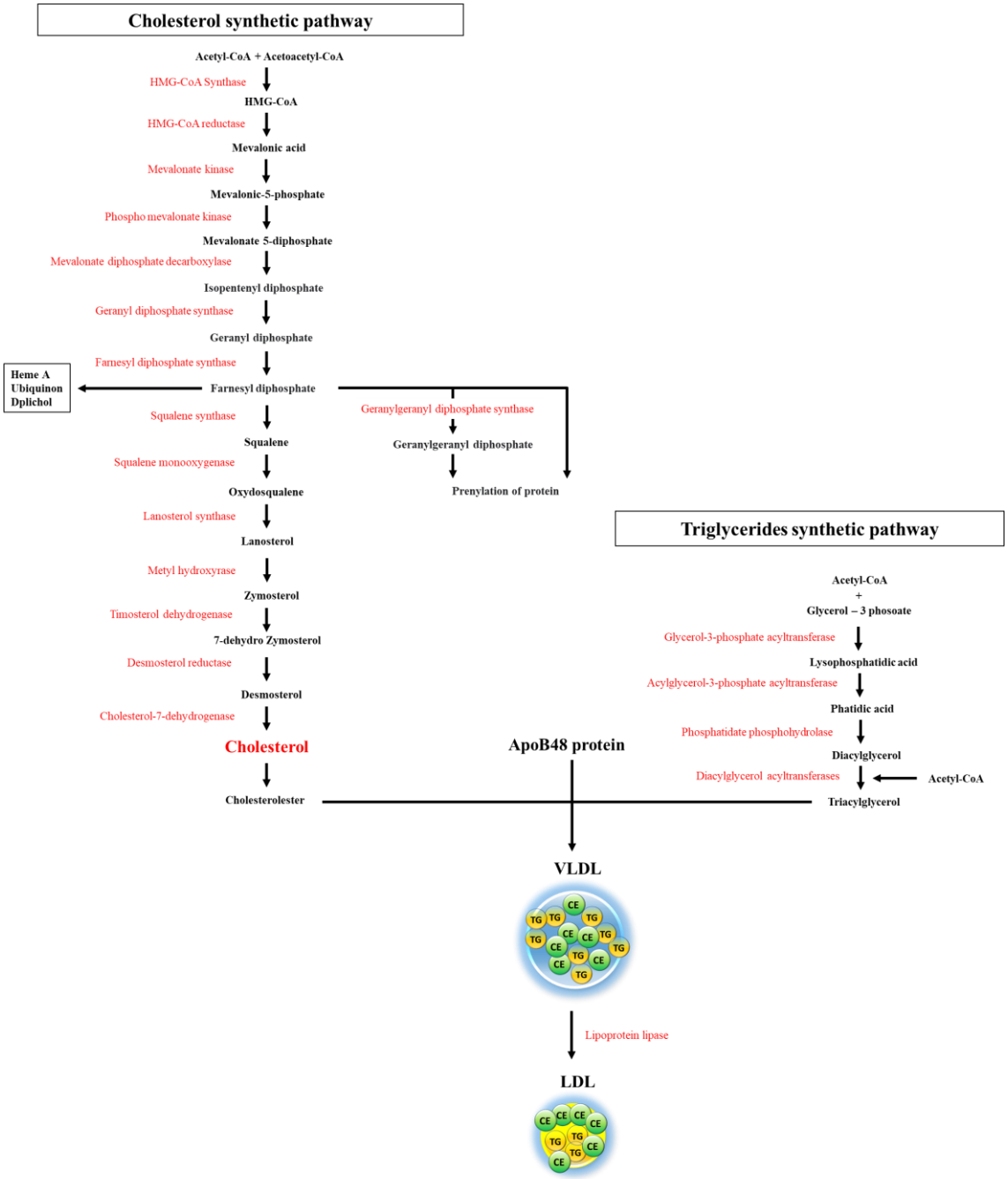


Figure 3 Cholesterol and triglyceride pathways for LDL cholesterol synthesis.

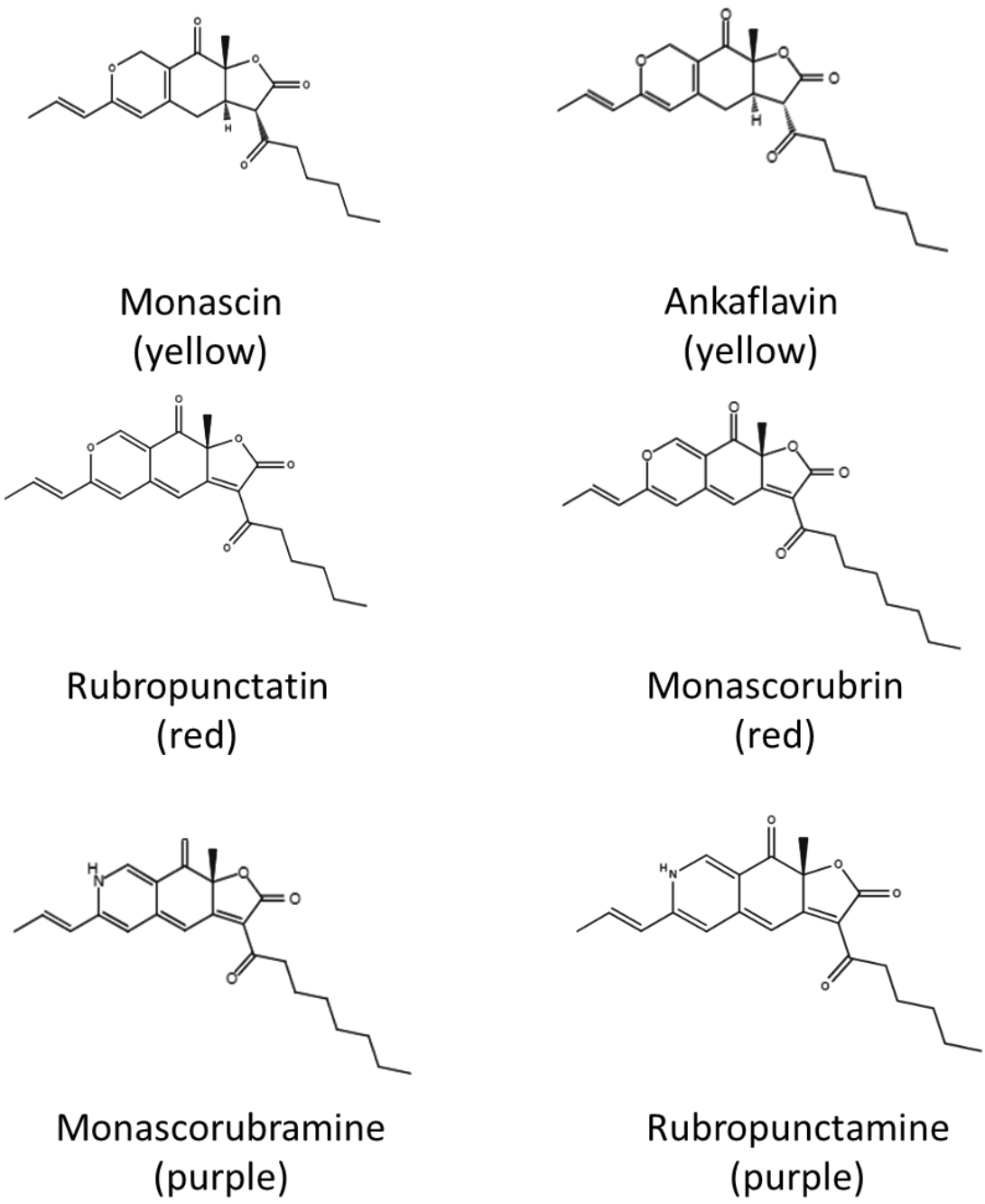


Figure 4 Chemical structures of major monascus azaphilone pigments.

Chapter2: Material and Method

2.1 Strain and culture conditions for LCMS analysis

Three *Monascus* species, specifically, *M. pilosus* NBRC 4520, *M. purpureus* NBRC 4478, and *M. rube*r NBRC 4483, were obtained from the National Institute of Technology and Evaluation in Japan. The *Monascus* were cultured in potato dextrose liquid medium using Potato Dextrose Broth (BD Difco, New Jersey,USA) at 30℃ for 7 days with 140 rpm shaking in TAITC BR-23FP. The cultures were collected and lyophilized at -80℃. Dry powder was 10mg sampling in a 1.5ml tube, in addition of MeOH 1ml, was subjected to ultrasonic treatment. The extracted sample was centrifuged at 15,000rpm, 25℃, for 10 minutes. The supernatant was collected and filtered through a 0.22μm filter (Merck, Darmstadt, Germany) to prepare an analytical sample. Samples were measured using a Shimadzu LCMS-8040 system (Shimadzu, Kyoto, Japan) with 300mm ODS MonoBis columns (Kyoto Monotech Co., Ltd., Kyoto, Japan). Each metabolite was estimated based on the *m/z* of each peak, referring to the *m/z* of the metabolites previously reported in *Monascus*. We measured three replicates for each species and applied two-dimensional hierarchical clustering to visualize their similarity, using the Euclidian distance of their profiles of the observed pigments concentration as a measure of similarity score and applying the Ward method.

2.2 Draft genome sequencing and assembly

Three types of *Monascus* cultured at 30℃ for 7 days in Potato Dextrose liquid medium were collected. Genomic DNA was individually isolated from the recovered cells and sequenced using the Illumina MiSeq (illumina, California, USA) paired-end library (read both ends at 300 bp and insert 500 inserts). The NGS data was acquired by Miseq at Axiohelix Co., Ltd. Approximately 8.5 million reads (around 5 Gb) for each sample were obtained and assembled using ABySS 2.0 de novo assembler [40]. We obtained 5000 to 10,000 assembled scaffolds for each samples and the N50 value of the total scaffolds were 133 Kb. The accumulated total length of the assembled contigs was 24.8 Mb, which is close to that of *M. purpureu*s NRRP 1596 genome (ATCC 16365)

with 23.4 Mb [38] and *M. ruber* NRRP 1597 (ATCC 13692) with 24.9 Mb [38]. To identify the gene-coding regions, the nucleotide sequence of the assembled scaffolds was annotated using DIAMOND, a high throughput BLASTX compatible sequence alignment algorithm [41]. The assembled sequences were also analyzed by BLASTed using the library of the whole UniProtKB/Swiss-Prot database [42]. Annotated genes of *M. purpureus* NRRP 1596 and M. ruber NRRP 1597 for were used for validation, with a cutoff E-value <1E-10. We further analyzed the genomes using antiSMASH pipeline [43] to extract the functional gene clusters such as PKS, in each *Monascus* species.

2.3 Analysis of secondary metabolite gene clusters

To evaluate the gene cluster related to secondary metabolic pathways from the resulting draft genome, it was used antiSMASH 4.0. A tree diagram was created using the BLAST alignment core using the secondary metabolic synthesis genes. Secondary metabolites synthesized from the secondary metabolite synthesis gene group predicted by antiSMASH 4.0 are summarized in a table. Furthermore, among the secondary metabolites, the gene clusters related to monacolin K, azaphyllone pigment, and citrinin have been reported in detail [44, 45, 46]. Using the gene sequences of monacolin K, azaphilone pigments, and citrinin as templates, homology analysis of genes and proteins was performed by BLAST.

Chapter3: Results and Discussion

3.1 LCMS analysis of *Monascus* cultures

Monascus species can produce several types of azaphilones, including nitrogenated azaphilones, N-glucosyl azaphilones, amino acid derivative azaphilones, and citrinins [47]. We cultivated the three *Monascus* species to compare the production of the pigments and their related metabolites using both agar and liquid medium, i.e., potato dextrose agar (PDA) and potato dextrose liquid (PDL) medium, which is the most frequently used culture medium for *Monascus* growth and metabolite production [48]. As shown in Figure5, distinct colony shapes and colors could be observed on PDA and in PDL among *M. pilosus*, *M. ruber*, and *M. purpureus*. In order to quantify the difference of the pigment contents, we analyzed the medium using LC-MS and identified 14 pigments in total. Figure6 showed the concentration of 14 metabolites quantified in three biological replicates in the three species as a heatmap with two-dimensional hierarchical clustering to display their similarity. This result confirmed that not all these metabolites are synthesized in the three species. The numbers of pigments commonly identified in each species are summarized as the Venn diagram (Fig. 7). Dehydromonacolin K, rubropunctatin, monascin, and ankaflavin 2 were commonly produced by all three *Monascus* species. Of the three species, *M. pilosus* produced the greatest number of pigments (12 in total; Fig. 7). Ten pigments, except monascorubramine, were produced by *M. pilosus*, while ankaflavin 1 and rubropunctamine were only produced by *M. pilosus*. Citrinin, a mycotoxin with nephrotoxic activity in mammals [49], was only produced by *M. purpureus*.

3.2 Biosynthetic pathway of detected metabolites

In order to understand the difference of the metabolites produced in these three species, we investigated their biosynthesis pathways in detail and illustrated in Fig. 8. It has been identified that the biosynthesis of the precursor of these metabolites, 1Hisochromenes are started from malonyl-CoA, in *Penicillium marneffei* and *M. ruber* [9, 28]. Citrinin polyketide synthase (PKS) converts the PKS-bound product citrinin [9]. The biosynthetic

pathway from malonyl-CoA to monacolins was also determined in *Aspergillus terreus*, *M. ruber*, and *M. purpureus* [50, 51]. It should be noted that PKS-bound products are acted upon by two different types of PKS enzymes – one is an enzyme to produce Monascus azaphilone pigments, which corresponds to the pathway from malonyl-CoA to 1H-isochromenes and nitrogenated azaphilones [9], and the other is citrinin polyketide synthase, which corresponds to the pathway from PKS-bound product to citrinin [52]. We also added the amount of the metabolites associated with each precursor as color bars. Among the three *Monascus* species, all eight metabolites related to 1Hisochromenes were only detected in *M. pilosus*. As for the other pathways, while citrinin was observed only in *M. purpureus*, Dehydromonacolin K, which is a precursor for monacolin K production, was detected in all three species. These resutls showed that productions of secondary metabolites are distinct among these three *Monascus* species. Azaphilone pigments related to 1H-isochromenes and nitrogenated azaphilones were observed among the three species, but azaphilone pigments related to ankaflavin 1 and rubropunctatin were observed in *M. pilosus*, while monascorubramine was detected in the other two species. Azaphilone pigments related to ankaflavin 1 and rubropunctatin were observed in *M. pilosus*, while monascorubramine was detected in the other two species.

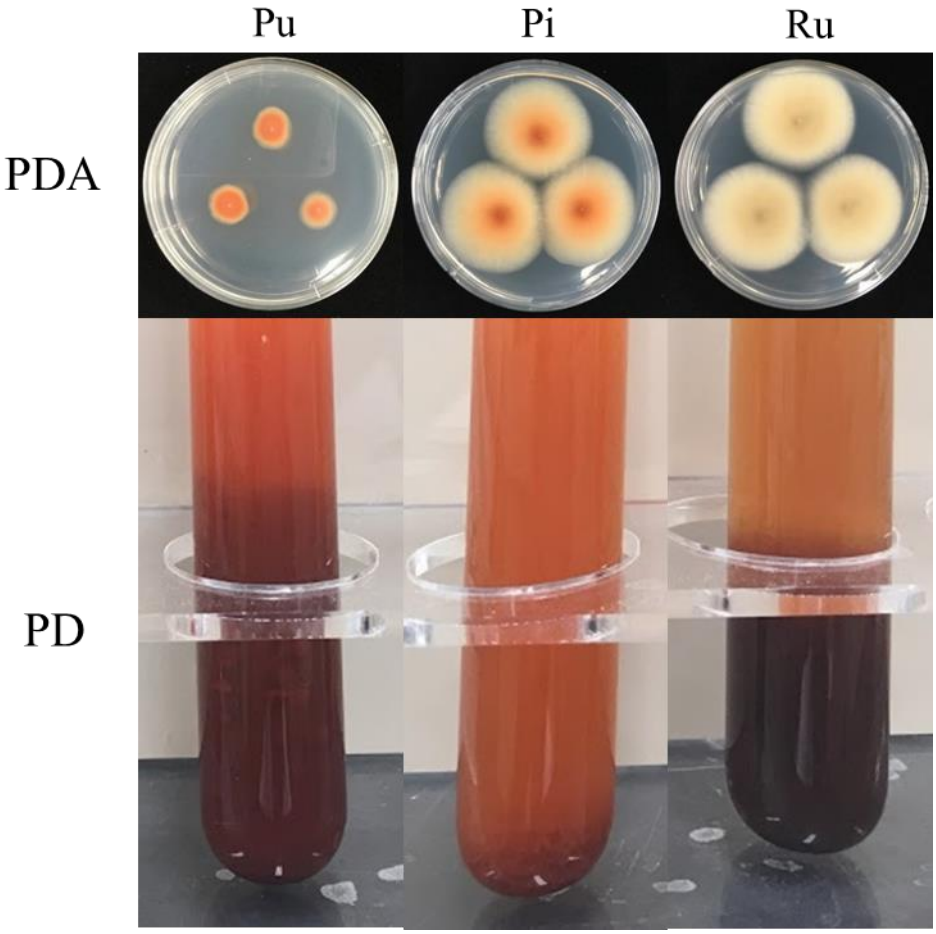


Figure 5　Three *Monascus* species for PD and PDA cultures at 30℃.

Pu: *Monascus purpureus* NBRC4478 Pi: *Monascus pilosus* NBRC4520 Ru: *Monascus ruber* NBRC4483

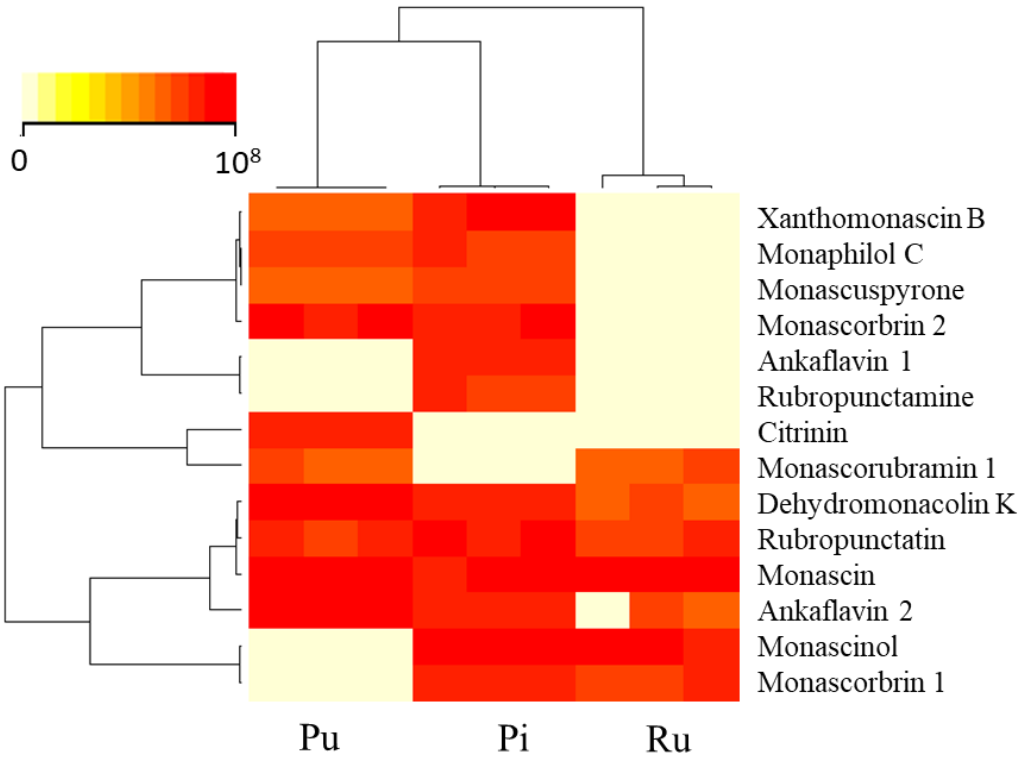


Figure 6 2D clustering of secondary metabolites quantities among the three species. Secondary metabolites in *Monascus* potato dextrose liquid culture obtained by LCMS analysis were comparatively quantified and two-dimensional clustered. Pu: *Monascus purpureus* NBRC4478 Pi: *Monascus pilosus* NBRC4520 Ru: *Monascus ruber* NBRC4483

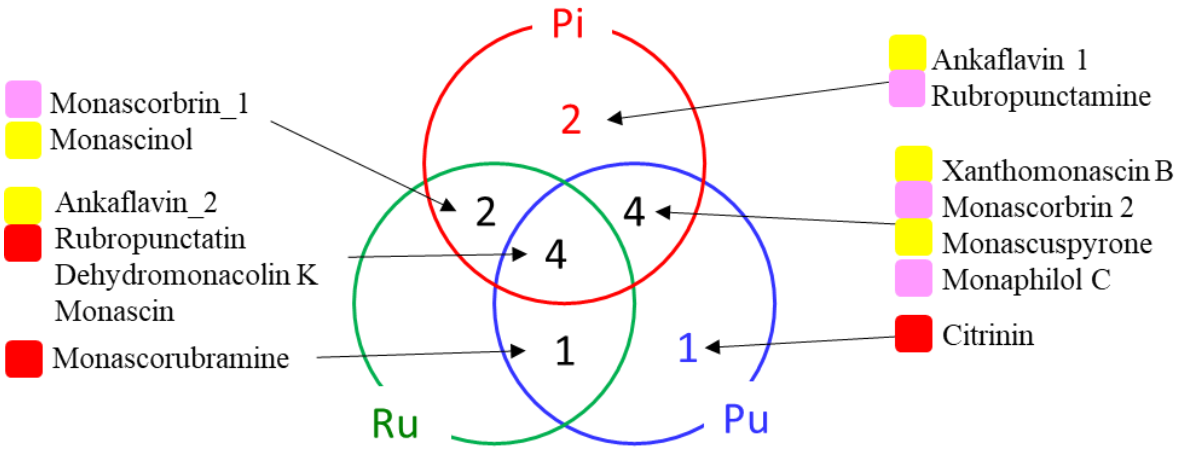


Figure 7 Venn diagrams of the metabolites observed in PDL culture.

The relationship between the metabolites detected by LCMS and the strains is shown on the Venn diagram. Pu: *Monascus purpureus* NBRC4478 Pi: *Monascus pilosus* NBRC4520 Ru: *Monascus ruber* NBRC4483

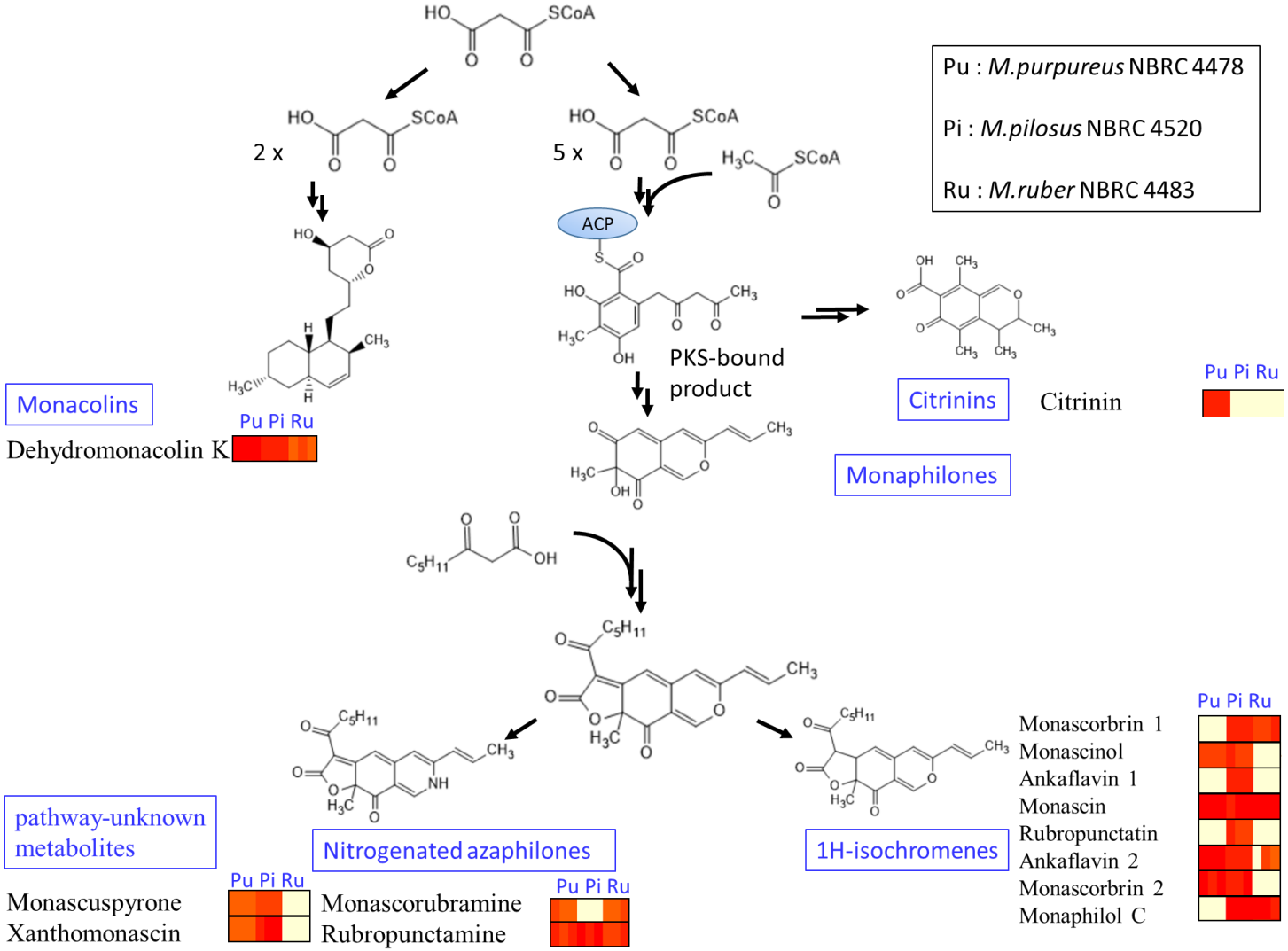


Figure 8 The metabolic pathways of five major groups. (i) monacolins, (ii) citrinins, (iii) monaphilines, (iv) 1H-isochromenes, and (v) nitrogenated azaphilones. The color bars represent the average concentration of metabolites in three replicates shown in Fig. 6.

3.3 Assembling and annotation of NGS data

Annotation was performed in Assembly By Short Sequences (ABySS) (http://www.bcgsc.ca/platform/bioinfo/software/abyss) using the data obtained from NGS. The result of the annotation is shown in Table 2. Figure 9 shows statistics on the length of the assembled sequence. The predicted genome size was 23 Mb. The sum of the contigs obtained this time was 24.3 Mb (105.6%). There was sufficient data for max length, and N50 was also > 100K. Therefore, the accuracy of assembly was expected to be high.

Table 2 Statistics of the assembled scaffolds results

	n	N50	max	sum
***M. purpureus* NBRC4478**	5287	133171	555966	23.5 Mbp
***M. ruber* NBRC4483**	9652	133065	441130	24.6 Mbp
***M. pilosus* NBRC4520**	10065	125733	441127	24.8 Mbp

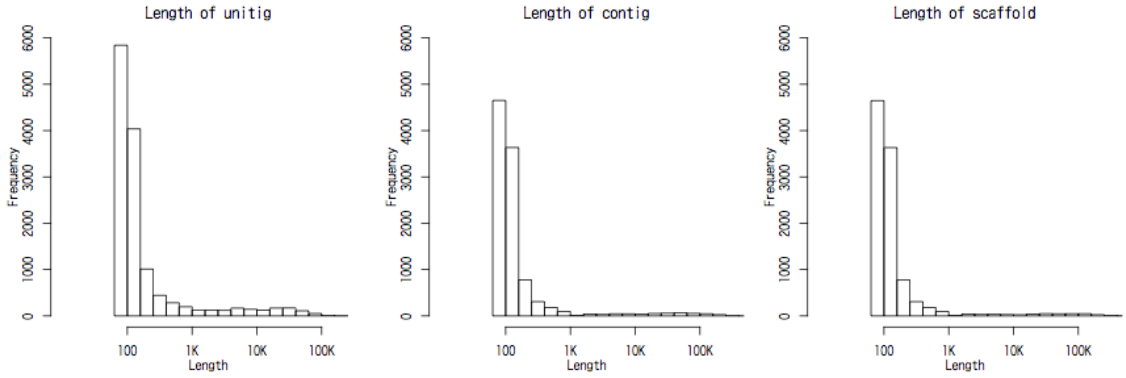


Figure 9 Distribution of the length of assembled sequences.

Next, the ORF region of the obtained genome was predicted using *Monascus ruber* NRRL1597 (https://genome.jgi.doe.gov/portal/MonrubNRRL1597_FD/) as the reference genome. The predicted ORF results are summarized in figure 10. From these results, the regions where the protein was predicted to be translated were 8,647 ORFs for *M.pilosus* NBRC4520, 8,646 ORFs for *M.ruber* NBRC4483, and 8,891 ORFs for *M.purpureus* NBRC4478. Of the ORFs of *Monascus ruber* NRRL 1597, 17 ORFs were predicted to be absent from any strain. In addition, the ORF regions of *M.pilosus* NBRC4520 and *M.ruber* NBRC4483 had a fairly high degree of agreement. On the other hand, it was revealed that *M. purpureus* NBRC4478 is different from *M.pilosus* NBRC4520 and *M.ruber* NBRC4483 and has an ORF region of 243 ORFs.

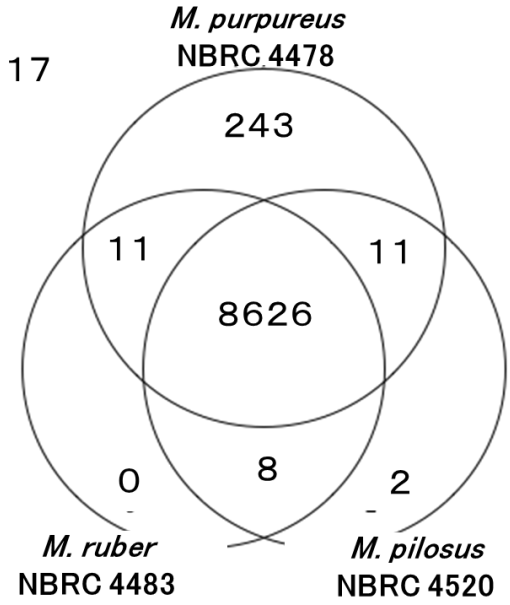
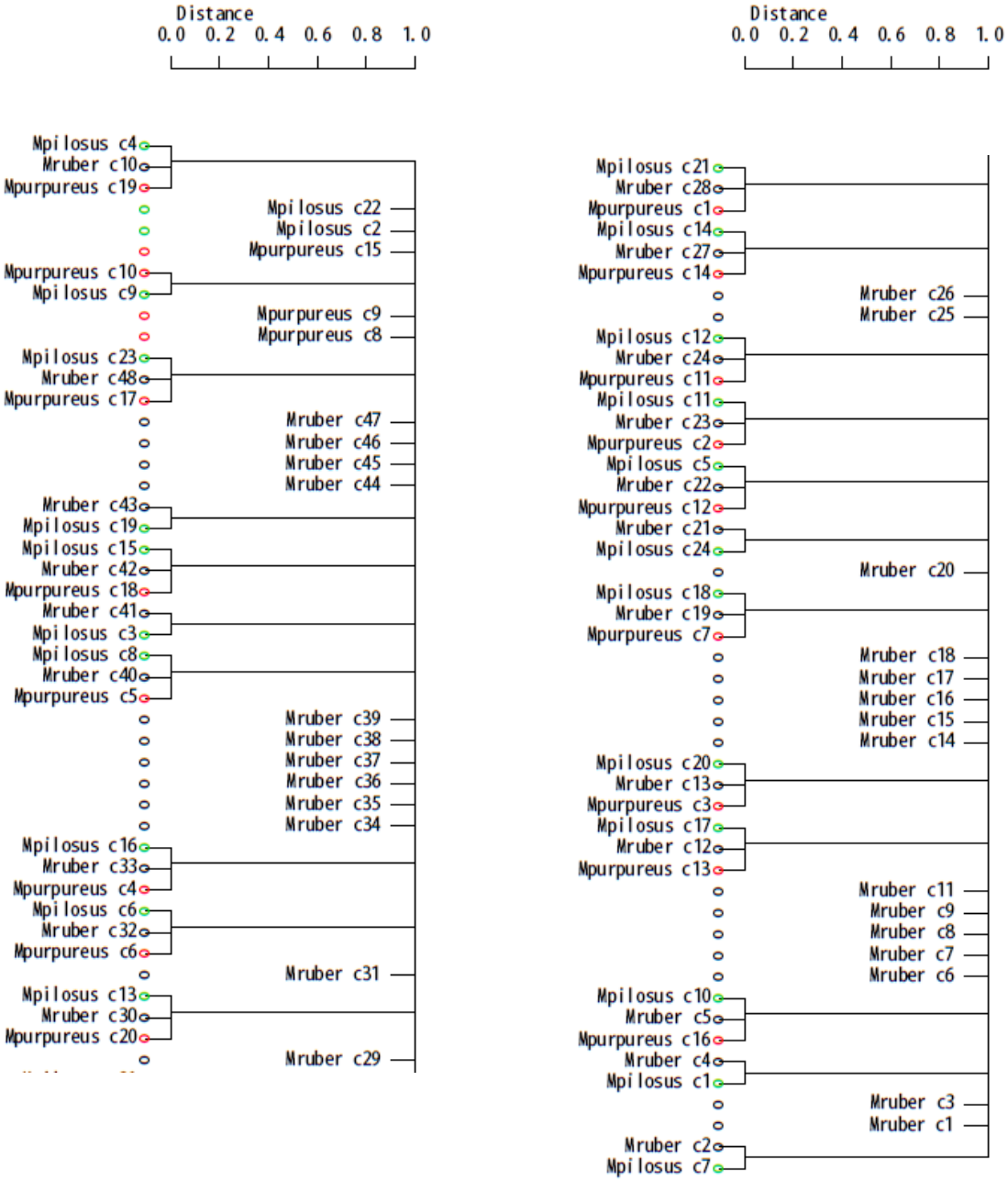


Figure 10　Venn diagram predicting the ORF of each strain using *Monascus ruber* NRRL 1597 as the reference genome.

3.4 Analysis of secondary metabolites gene clusters

The obtained draft genome was predicted for secondary metabolite genes using antiSMASH 4.0. As a result, 24 secondary metabolite genes were predicted for *M.pilosus* NBRC4520, 48 genes for *M.ruber* NBRC4483, and 20 genes for *M.purpureus* NBRC4478. A tree diagram of the obtained secondary metabolite gene cluster was created using the BLAST tool (Table 3). 32 gene clusters were grouped. In addition, 22 gene clusters were independent. Gene clusters with the same genetic makeup were integrated into a single group. The genetic coding region was predicted using a BLASTX search against the UniProtKB / Swiss-Prot database (E value <1.0 × 10-10). The gene clusters of 54 groups (Table 4) are shown in the Venn diagram (Fig. 11). Gene clusters were characterized as secondary metabolite or ABC transporter clusters based on their similarity to known genes [53]. ABC transporters are associated with PKS and NRPS gene clusters of several fungi and are involved in the export of corresponding secondary metabolites [54]. In addition, the compounds and functions predicted to be metabolized from the genes predicted from BLASTX and fungal antiSMASH 5.20 are summarized (Fig. 12-15). Many of the compounds predicted from antiSMASH have not been reported in *Monascus*. From these results, it is expected that *Monascus* produce an unknown secondary metabolite.

Table 3 Dendrogram of secondary metabolite biosynthetic gene clusters observed in three *Monascus* species. The secondary metabolism gene clusters of *M. purpureus* NBRC 4478, *M. pilosus* NBRC 4520, and *M. ruber* NBRC 4483 estimated in this study are represented by red circles, green circles, and black circles.



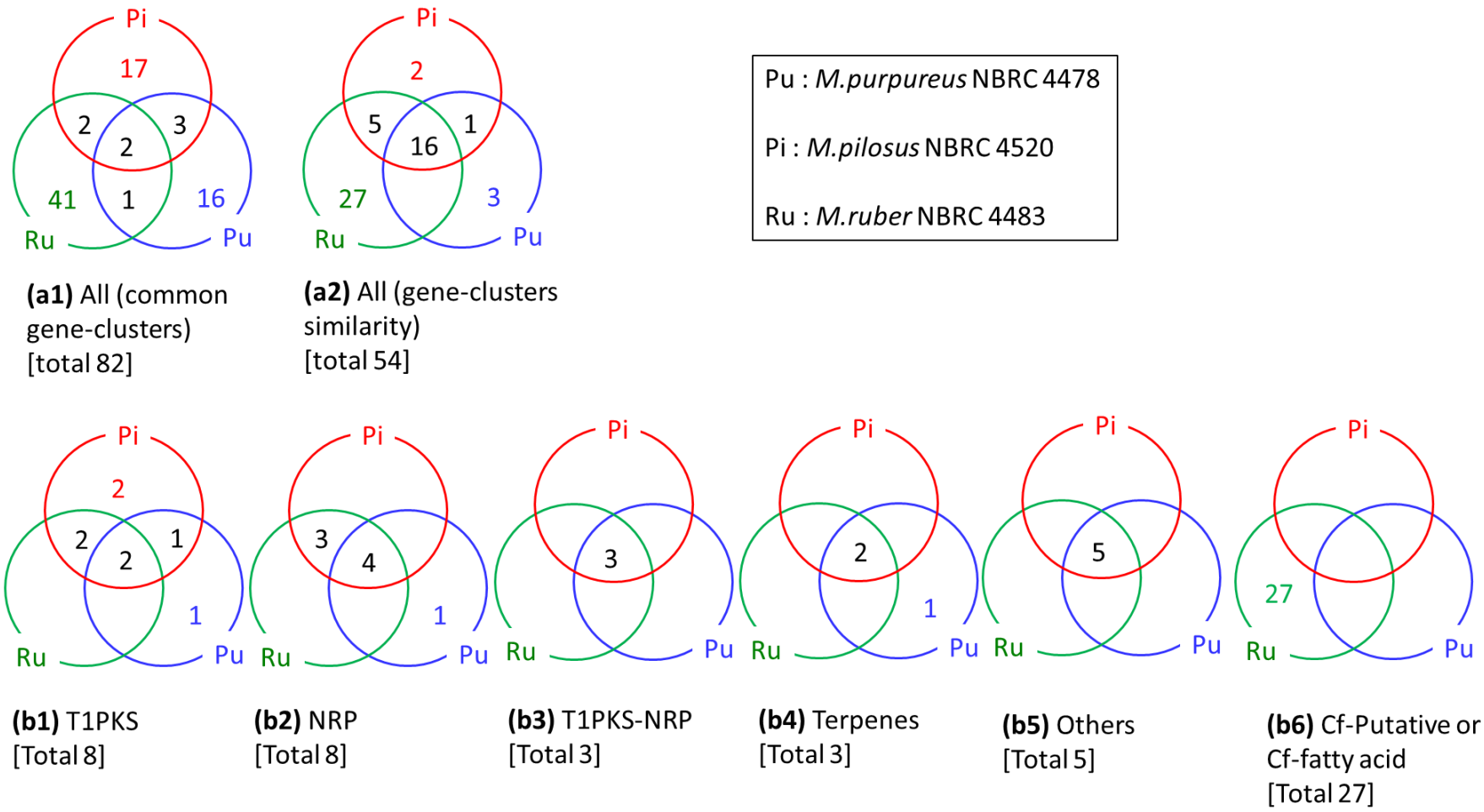


Figure 11 Venn diagrams of secondary metabolite biosynthetic gene clusters observed in three *Monascus* species. (a1):Venn diagram classifying 82 secondary metabolic synthesis gene clusters of three *Monascus* species. (a2):Venn diagram classifying 82 secondary metabolic synthesis gene clusters of three *Monascus* species based on DNA sequence homology. The total number of gene clusters was 54. Venn diagram classifying 54 secondary metabolic synthesis gene clusters of three *Monascus* species based on DNA sequence homology: T1PKS (b1), NRPS (b2), T1-PKS-NRPS (b3), Terpenes (b4), Others (b5), Cf-Putative or Cf-fatty acid (b6)

Table 4 Classification of gene clusters (ID1-54) corresponding to the dendrogram in Figure 11.

ID	Type of GCs	Pi	Ru	Pu	Secondary metabolic pathways detected in DNA sequence homology	ATP-binding cassettes	Identity level
1	t1pks	1	4	-	Narbonolide/10-deoxymethynolide (*pikAI – AIV*), Phthiocerol/phenolphthionocerol (*ppsA - E*)	-	**
2	t1pks	2	-	-	Phthiocerol/phenolphthionocerol (*ppsA - E*)	-	-
3	t1pks	18	19	7	Azaphilone*, Lovastatin (LOVB, LOVF) , Narbonolide/10-deoxymethynolide (*pikAI – AIV*), Phthiocerol/phenolphthionocerol (*ppsA - E*)	-	-
4	t1pks	22	-	-	-	-	*
5	t1pks	23	48	17	Lovastatin (LOVB, LOVF) , Narbonolide/10-deoxymethynolide (*pikAI – AIV*), Phthiocerol/phenolphthionocerol (*ppsA - E*)	-	*
6	t1pks	19	43	-	Monacolin K*, Compactin, Lovastatin (LOVA, LOVB)	-	*
7	t1pks	-	-	8	Citrinin*, Narbonolide/10-deoxymethynolide (*pikAI–AIV*), Phthiocerol/phenolphthionocerol (*ppsA-C, E*)	-	*
8	t1pks	9	-	10	Byssochlamic acid*, Narbonolide/10-deoxymethynolide (*pikAI–AIV*), Phthiocerol/phenolphthionocerol (*ppsA-C, E*)	-	*
9	nrps	4	10	19	Neosartoricin*, Fengycin (*fenA–E*), Surfacin(*srfAA-AC*)	-	**
10	nrps	13	30	20	Brevianamide F (*FTMA*)	ABCB	**
11	nrps	7	2	-	Fengycin (*fenA-E*), Surfacin (srfAA-AC)	ABCC	**
12	nrps	11	23	2	Fengycin (*fenA-D*)	ABCC	**
13	nrps	5	22	12	Fengycin (*fenA-D*)	-	*
14	nrps	24	21	-	Fengycin (*ppsA-D*), surfacing (*srfAA-AC*)	ABCB	*
15	nrps	3	41	-	Fengycin (*fenA–D*)	-	*
16	nrps	-	-	9	Fengycin (*fenB, D*), Ferricrocin (*SIDC, SIDD*)	-	-
17	t1pks-nrps	8	40	5	NG-391*, Fengycin (*fenA–E*), Surfacin (*surAA-AC*)	ABCC	**
18	t1pks-nrps	14	27	14	Gramicidin (*grsA-B*), Fengycin (*fenA–E*), Surfacin (*surAA-AC*)	-	*
19	t1pks-nrps	20	13	3	Lovastatin (*LOVB, LOVF*), Fengycin (*fenA-E*), Surfactin (*srfAA-AC*)	ABCB	*

Table 4 (continued)

ID	Type of GCs	Pi	Ru	Pu	Secondary metabolic pathways detected in DNA sequence homology	ATP-binding cassettes	Identity level
20	terpene	12	24	11	Fernesyl-diphosphate (*FDFT1*)	-	**
21	terpene	17	12	13	Lupeol (*LUP1,2,4,5*), Arabidiol (*PEN1*), Tinucalladienol (*PEN3*), seco-amyrin (*PEN6*)	-	*
22	terpene	-	-	15	-	-	-
23	others	15	42	18	Fengycin (*fenA, C, E*), Surfacin (srfAA-AB)	ABCF	**
24	others	6	32	6	Kinesin (KIDFC1-3)	-	*
25	others	10	5	16	Abscisic aldehyde (AAO1-4), Fengycin (*fenA, B, E*), Surfactin (*srfAA, AB*)	ABCB	*
26	others	16	33	4	Histidinol (*hisD, IE*)	-	*
27	others	21	28	1	-	-	*
28	cf-putative	-	1	-	-	-	-
29	cf-putative	-	3	-	Palmitin (*ZDHHC*)	-	-
30	cf-putative	-	6	-	Palmitin (*ZDHHC4)*	-	-
31	cf-putative	-	7	-	-	-	-
32	cf-putative	-	8	-	-	-	-
33	cf-putative	-	9	-	-	-	-
34	cf-putative	-	11	-	-	-	-
35	cf-putative	-	14	-	Serine, Threonine (*PP1C, 2C, 4C, 6C*)	-	-
36	cf-putative	-	15	-	-	ABCG2	-
37	cf-putative	-	16	-	-	-	-
38	cf-putative	-	17	-	-	-	-
39	cf-putative	-	18	-	Mannan (*ANPI, MNN9*)	-	-
40	cf-putative	-	20	-	-	-	-
41	cf-putative	-	25	-	-	-	-
42	cf-putative	-	29	-	-	-	-
43	cf-putative	-	31	-	-	-	-
44	cf-putative	-	34	-	-	-	-
45	cf-putative	-	36	-	-	-	-
46	cf-putative	-	37	-	-	-	-
47	cf-putative	-	38	-	-	-	-
48	cf-putative	-	39	-	Lovastatin (*LOVB-G*), Phthiocerol/Phenolphthiocerol (*ppsA,C*)	-	-
49	cf-putative	-	44	-	-	-	-
50	cf-putative	-	45	-	-	-	-
51	cf-putative	-	46	-	-	-	-
52	cf-putative	-	47	-	-	-	-
53	cf_fatty_acid	-	26	-	-	-	-
54	cf_fatty_acid	-	35	-	Fatty acid (*FAS1,2*)	-	-

The type of GCs was determined using antiSMASH software. Pi, Ru, and Pu represent the cluster ID in Table 4. Red numbers denotes identical gene organization. The secondary metabolic pathways represent the secondary metabolite information based on DNA sequence homology. Type of ATP-binding cassettes detected in individual groups is represented as ATP-binding cassettes. Gene-cluster groups with both identical gene organization and high DNA sequence similarity are denoted by '**' and groups with only high DNA sequence similarity are denoted by '*'. Two-letter abbreviations use used for the Venn diagrams: Pi, *M. pilosus* NBRC 4520; Ru, *M. ruber* NBRC 4483; Pu, *M. purpureus* NBRC 4478.

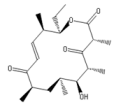
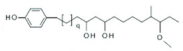
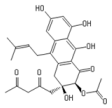
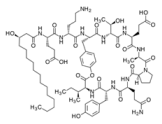
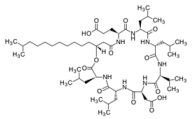
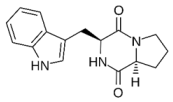
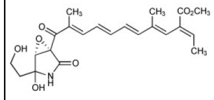
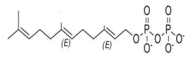
ID	compound	structure	molecular weight	role of compound	reference
1	Narbonolide		352.5	Intermediate of the antibiotic picromycin in favor of Streptomyces venezuelae	Gupta *et al*. [55]
1	Phenol phthiocerol		—	Glycolipids that make up the extracellular antigen of mycobacterium	Mamadou DAFFE *et al*. [56]
9	Neosartoricin		484.5	T cell antiproliferative activity	Yit-Heng Chooi *et al*. [57]
9 11 17	Fengycin		1463.7	Antibacterial activity of filamentous fungi	Nongnuch Vanittanako *et al*. [58]
9 11 17	Surfactin		1036.3	Red blood cell lysis, Inhibition of blood clot formation, dissolution of bacterial spheroplasts and protoplasts, snd Inhibits cyclic 3', 5-monophosphate diesterase	Nikhil S *et al*. [59]
10	Brevianamide F		283.3	Antibacterial activity against methicillin-resistant Staphylococcus aureus	Francielly M *et al*. [60]
17	NG-391		417.5	Anti-tumor	Markus Bohnert *et al*. [61]
20	Fernesyl diphosphate		382.3	Intermediate of the mevalonate pathway	Anna *et al*. [62]

Figure 12 List of compounds and functions synthesized from secondary metabolite genes predicted from BLASTX.

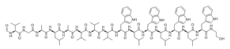
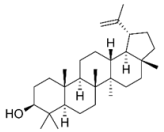
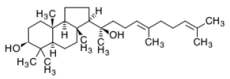
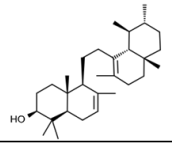
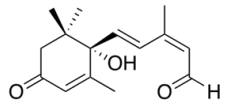
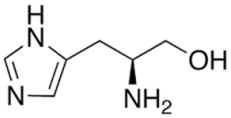
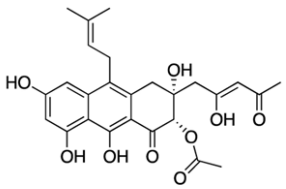
ID	compound	structure	molecular weight	role of compound	reference
18	Gramicidin A		1882.2	Channels for passing water and ions	Zhanhu Sun *et al*. [63]
21	Lupeol		426.7	Wound healing, diabetes , cardiovascular disease, kidney disease, and arthritis	Nitika Sharma *et al*. [64]
21	Arabidiol		444.7	Metabolites isolated from Arabidopsis	Dorianne A. Castillo and Reza Sohrabi *et al*.[65,66]
21	α-seco-amyrin		426.7	Secondary metabolites synthesized by plants	Masaaki Shibuya *et al*. [67]
25	Abscisic aldehyde		248.3	Intermediate of abscisic acid synthesis pathway	Shahid Ali and Christopher D. Rock *et al*.[68, 69]
26	Histidinol		141.1	Anti-tumor	R. C. Warringto and Nagai Atsuko *et al*. [70, 71]

Figure 12 (continued)

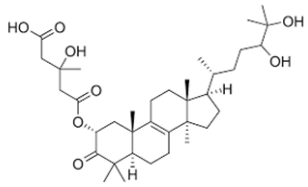
(A)

M.ruber NBRC4483						
Region	Type	From	To	Most similar known cluster	Type of synthesize clusters	Similarity
Region 23.1	NRPS	23,719	69,152	-	-	-
Region 46.1	T1PKS	1	24,079	neosartoricin B	Polyketide	40%
Region 47.1	NRPS-like	19,272	64,129	-	-	-
Region 98.1	NRPS	1	41,063	-	-	-
Region 104.1	terpene	1	19,704	clavaric acid	Terpene	100%
Region 114.1	T1PKS,NRPS	1	28,447	-	-	-
Region 203.1	T1PKS	20,586	55,671	ankaflavin / monascin / rubropunctatine / monascorubrin	Polyketide:Iterative type I	33%
Region 291.1	NRPS	70,326	130,680	-	-	-
Region 295.1	NRPS	30,780	80,075	-	-	-
Region 315.1	NRPS	1	45,174	-	-	-
Region 340.1	terpene	72,304	93,866	squalestatin S1	Terpene	40%
Region 364.1	NRPS,T1PKS	15,145	100,934	-	-	-
Region 370.1	betalactone	106,537	135,048	-	-	-
Region 371.1	NRPS-like	38,474	81,780	-	-	-
Region 386.1	NRPS	2,631	32,574	-	-	-
Region 399.1	NRPS-like	186,371	212,111	-	-	-
Region 402.1	NRPS-like	12,393	55,635	-	-	-
Region 418.1	NRPS,T1PKS	137,971	181,918	NG-391	NRP + Polyketide	33%
Region 427.1	NRPS	209,425	254,749	-	-	-
Region 433.1	NRPS-like	81,653	125,474	-	-	-
Region 434.1	T1PKS	141,976	174,148	monacolin K	Polyketide	22%
Region 437.1	betalactone	88,341	111,547	-	-	-
Region 457.1	T1PKS	63,124	95,561	-	-	-

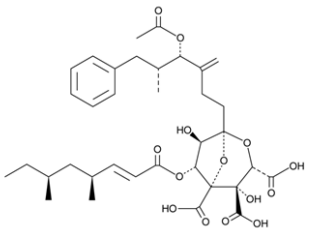
(B)



neosartoricin B



clavaric acid



squalestatin S1

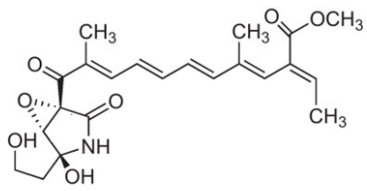


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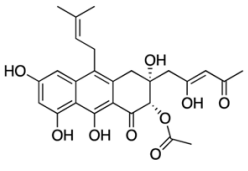
Figure 13 Prediction of compounds synthesized with secondary metabolite genes using Fungal antiSMASH 5.20 by *Monascus ruber* NBRC4483. (A) List of predicted secondary metabolites gene clusters. (B) Predicted secondary metabolites.

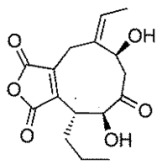
(A)

M.pilosus NBRC4520						
Region	Type	From	To	Most similar known cluster	Type of synthesize clusters	Similarity
Region 29.1	T1PKS	1	24,246	neosartoricin B	Polyketide	40%
Region 73.1	T1PKS	1	24,108	-	-	-
Region 86.1	NRPS	4,243	43,392	-	-	-
Region 87.1	NRPS	1	41,063	-	-	-
Region 114.1	NRPS	1	35,952	-	-	-
Region 123.1	NRPS-like	1	25,409	-	-	-
Region 149.1	NRPS	32,893	78,332	-	-	-
Region 203.1	T1PKS,NRPS	1	44,044	NG-391	NRP + Polyketide	33%
Region 232.1	T1PKS	255,436	286,306	cornexistin	Polyketide	9%
Region 254.1	NRPS-like	19,285	64,142	-	-	-
Region 268.1	NRPS	1	49,778	-	-	-
Region 322.1	betalactone	13,605	42,117	-	-	-
Region 328.1	terpene	38,952	60,514	squalestatin S1	Terpene	40%
Region 354.1	NRPS	2,630	32,572	-	-	-
Region 356.1	T1PKS,NRPS	93,158	178,645	-	-	-
Region 363.1	NRPS-like	49,808	93,629	-	-	-
Region 374.1	NRPS-like	64,234	107,476	-	-	-
Region 384.1	terpene	239,135	263,684	-	-	-
Region 386.1	T1PKS	3,374	30,897	ankaflavin / monascin / rubropunctatine / monascorubrin	Polyketide:Iterative type I	33%
Region 386.2	T1PKS	245,958	278,153	monacolin K	Polyketide	22%
Region 396.1	T1PKS,NRPS	1	35,090	-	-	-
Region 402.1	NRPS-like	43,184	86,490	-	-	-
Region 403.1	T1PKS	1	40,710	-	-	-
Region 408.1	T1PKS	140,231	187,336	-	-	-
Region 415.1	NRPS	42,475	102,829	-	-	-
Region 422.1	betalactone	154,543	177,749	-	-	-

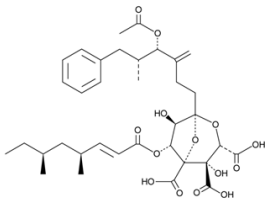
(B)



neosartoricin B



cornexistin



squalestatin S1

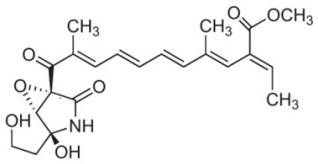


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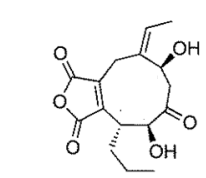
Figure 14 Prediction of compounds synthesized with secondary metabolite genes using Fungal antiSMASH 5.20 by *Monascus pilosus* NBRC4520. (A) List of predicted secondary metabolites gene clusters. (B) Predicted secondary metabolites.

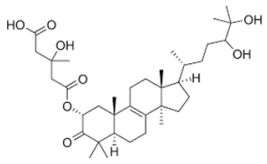
(A)

M.purpureus NBRC4478						
Region	Type	From	To	Most similar known cluster	Type of synthesize clusters	Similarity
Region 73.1	NRPS-like	61	26,525	-	-	-
Region 80.1	betalactone	1	26,518	-	-	-
Region 94.1	NRPS	1	47,215	-	-	-
Region 113.1	T1PKS,NRPS	35,653	87,810	-	-	-
Region 188.1	NRPS-like	10,565	55,069	-	-	-
Region 208.1	NRPS,T1PKS	240,669	292,506	NG-391	NRP + Polyketide	50%
Region 218.1	NRPS-like	54,904	98,587	-	-	-
Region 247.1	T1PKS	119,334	167,618	ankaflavin / monascin / rubropunctatine / monascorubrin	Polyketide:Iterative type I	37%
Region 249.1	T1PKS	1,800	49,133	Citrinin	Polyketide:Iterative type I	56%
Region 254.1	NRPS	436,747	469,196	-	-	
Region 256.1	T1PKS	50,979	99,661	Cornexistin	Polyketide	14%
Region 261.1	terpene	108,492	130,119	squalestatin S1	Terpene	60%
Region 268.1	NRPS	8,223	62,586	-	-	-
Region 272.1	betalactone	39,263	63,315	-	-	-
Region 274.1	terpene	19,513	36,609	clavaric acid	Terpene	100%
Region 287.1	NRPS,T1PKS	36,760	117,950	-	-	-
Region 289.1	terpene	3,982	18,506	aspterric acid	Polyketide	75%
Region 290.1	NRPS-like,NRPS	3,077	72,645	-	-	-
Region 303.1	T1PKS	117,625	165,031	-	-	-
Region 311.1	NRPS-like	17,479	61,300	-	-	-
Region 314.1	NRPS	1	41,967	-	-	-
Region 315.1	NRPS	1	26,051	-	-	-

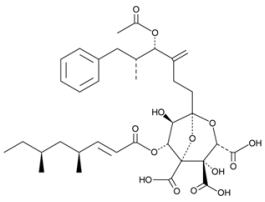
(B)



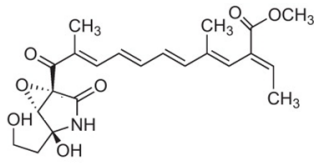
cornexistin



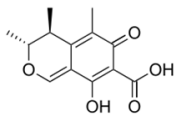
clavaric acid



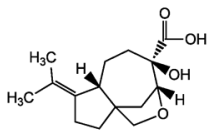
squalestatin S1



NG-391



citrinin



aspterric acid

Figure 15 Prediction of compounds synthesized with secondary metabolite genes using Fungal antiSMASH 5.20 by *Monascus purpureus* NBRC4478. (A) List of predicted secondary metabolites gene clusters. (B) Predicted secondary metabolites.

3.5 Comparative analysis of monacolin, azaphilone pigment and citrinin gene clusters

We compared the peptide sequences of citrinin biosynthetic genes in the three *Monascus* species with those in *M. purpureus* reported by Shimizu *et al*. [7] and Chen *et al*. (Fig.16) [73]. There are six genes associated with the citrinin biosynthetic pathway, consisting of pksCT (encoding polyketide synthase, PksCT/CitS), mpl1 (a serine hydrolase, CitA), mpl2 (an iron II oxidase, CitB), mpl7 (an oxidreductase, CitC), mpl4 (encoding an aldehyde dehydrogenase, CitD), and mpl6 (a short chain dehydrogenase, CitE). Table 5 shows the homologous regions of individual genes that aligned with the reference genes (E-value < 10− 44). Two proteins, CitB and CitS, were shorter in *M. ruber* and *M. pilosus* than the reference sequences. However, the same proteins were conserved in *M. purpureus* compared with the reference sequences. It suggests that the citrinin biosynthetic genes, mpl2 (CitB), and pksCT (CitS) were incomplete in *M. pilosus* NBRC 4520 and *M. ruber* NBRC 4483, and consequently, citrinin production is blocked in these species. Monacolin K was first isolated from the medium of *M. ruber* [5] and its biosynthesis pathway was determined, in *M. pilosus* (BCRC 387072), composed of nine enzymes that have a high level of homology with genes in the monacolin K biosynthetic gene cluster of *Aspergillus terreus* (Fig.16) [74]. Three strains of *M. purpureus*, specifically NRRP 1596, YY-1 (an industrial strain), and KACC 42430 (a laboratory strain), lack an intact monacolin K gene cluster [75]. In the present study, we also examined the monacolin gene clusters in three Monascus species. As shown in Table 6, all 9 Mok genes, especially MokC, MokD, and MokF, were shorter in *M. purpureus* NBRC 4478 than *M. pilosus* NBRC 4520 and *M. ruber* NBRC 4483. Thus, *M. purpureus* NBRC 4478 also lacks the complete monacolin K gene sequence. Furthermore, comparison of a 8144 bp region, in which a gene cluster of Monascus azaphilone synthases was localized, revealed that *M. pilosus* and *M. purpureus* can be clearly distinguished at the nucleotide level. In addition, *M. ruber* NBRC 4483 and NRRP 1597 have highly similar DNA sequences with *M. pilosus*; however, *M. ruber* JF83291.6 has highly similar DNA sequences with *M. purpureus* (Table 7).

Chapter4: Discussion

The three *Monascus* species examined in the present study are commonly used for food fermentation in the cuisine of East Asian countries [4,9,76]. Citrinin, a nephrotoxic agent, was reportedly produced in *M. purpureus* but not in *M. pilosus* [77, 78, 79]. This is corroborated by the present results from the metabolome and genome analyses revealing that citrinin biosynthetic pathways in *M. pilosus* were incomplete compared with those from *M. purpureus*. The three *Monascus* species can produce ubiquitous and species-specific pigment-related compounds (Fig.8 and Fig.17). Analysis of gene-organization revealed 54 greatly diverged gene clusters in the three *Monascus* species studied (Fig.11a2). Furthermore, comparison of a 8144 bp region, in which a gene cluster of Monascus azaphilone synthases was localized, revealed that *M. pilosus* and *M. purpureus* can be clearly distinguished at the nucleotide level. In addition, *M. ruber* NBRC 4483 and NRRP 1597 have highly similar DNA sequences with *M. pilosus*; however, *M. ruber* JF83291.6 has highly similar DNA sequences with *M. purpureus* (Table 6). Though in some phylogenetic studies [80, 81] *M. pilosus* and *M. ruber* were not distinguished, in our analysis, their phenotypes distance clearly distinct. On the other hand, taking the pigment biosynthetic gene clusters into consideration, *M. pilosus* and *M. purpureus* should be defined as different groups. Thus, based on the findings of the present study, the *Monascus* species studied here can be classified into two groups: (i) the *M. pilosus* clade and (ii) the *M. purpureus* clade. And the results shown in Table 6 suggests that there may some *M. rube*r strains, which can be related with each clade.

The mycotoxin citrinin is produced by various *Penicillium*, *Aspergillus*, and *Monascus* species [73, 78, 79]. Previously studied *M. purpureus* strains (ATCC 16365 in Java, 16379 in Taiwan, IFO 30873, and DSM 1379 by Chen and Ostry [73, 82]; YY-1 by Yang [38] can produce citrinin as a secondary metabolite. However, among the *Monascus* species, two *M. pilosus* strains (BCRC 38072 in Taiwan by Chen [73]; NBRC 4520 in this study) cannot produce citrinin. Interestingly, several previously reported *M. ruber* strains, particularly ATCC 16246, 16378, 16366, 18199, 16371, and 18199 by Chen [73], AUMC 4066 (CBS109.07) and AUMC 5705 by Moharram [83], NRRP 1597 by Kwon [75], and NBRC 4483 in this study, lack citrinin production activities, but other strains, such as

Tiegh by Ostry [82] and ATCC 96218 by Hajjaj [84] have the potential to produce this secondary metabolite. Thus, *M. ruber* can be classified into citrinin-producing and non-citrinin producing types. Based on the comparison of citrinin biosynthetic proteins, the former type might correspond to *M. purpureus* strains and the latter to *M. pilosus* strains.

In the analysis of the monacolin K gene cluster (Fig.16), four *M. purpureus* strains, specifically NRRP 1596, YY-1, KACC 42430 [75], and NBRC 4478 (in this study), lack an intact monacolin K gene cluster. By contrast, *M. pilosus* NBRC 4520 and *M. ruber* NBRC 4483 have a complete set of monacolin K gene clusters. Thus, it should be noted that *M. pilosus* NBRC 4520 and *M. ruber* NBRC 4483 can produce monacolin K but lack a complete set of citrinin biosynthetic gene clusters.

The classification of strains according to the two-clade groups designated as (i) *M. pilosus* and (ii) *M. purpureus* may play an important role in the food industry and industrial field through the improved utilization of *Monascus* species. However, in view of food safety, we need to confirm whether the toxins produced by some *Monascus* strains exist in the genome or metabolome. Metabolites are generally classified into primary metabolites that are essential for growth and reproduction and secondary metabolites that are usually involved in mechanisms for ecological adaptation but are not essential to regular cellular processes. Metabolic pathways can be divided into two types: one is the general pathway shared by most fungi and the other are specialized pathways that have evolved in response to specific ecologies of certain lineages and are consequently more narrowly distributed at the taxonomic level. The citrinin pathway belongs to the former as it is present in many *Penicillium*, *Aspergillus*, and *Monascus* species [77, 78, 79]. However, the biosynthetic gene cluster of Monascus azaphilone pigments is limited in the *Monascus* genera. The biosynthetic process of secondary metabolites forms a cluster or non-clustered gene organization that is integral to the entire spectrum of fungal ecological strategies (e.g., saprotrophic, pathogenic, and symbiotic). Gene duplication (GD) is often implicated in the evolution of fungal metabolism [85]. A second source of metabolic gene innovation in fungi is horizontal gene transfer (HGT), which includes xenobiotic catabolism [86], toxin production [87], and degradation of plant cell walls [88]. GD and HGT were more frequently found in clustered genes than in their non-clustered counterparts [89]. In the biosynthetic gene clusters of Monascus azaphilone pigments and

citrinin, the common trends in the strains of the three *Monascus* species are explained by the suggested *M. pilosus* and *M. purpureus* clades, whereas *M. ruber* has either *M. pilosus* or *M. purpureus* characteristics. *Monascus*-specific diverged pigments may have evolved because of GD and HGT events, resulting in the creation of clustered genes in their genomes; thus, a large number of gene clusters was observed (Table 1). Chemotaxonomy, including pigment production, is the most useful way to study the divergence of *Monascus* genera. Here, we compared the PKS responsible for the biosynthesis of azaphilone pigment from three *Monascus* species (*M. pilosus*, *M. purpureus* and *M. ruber*) and six strains. More genome sequences of *Monascus* species will need to be determined to better understand the production of secondary metabolites in these organisms.

In this study, the complete draft genome sequences of *M. pilosus* NBRC 4520, *M. purpureus* NBRC 4478, and *M. ruber* NBRC 4483 were obtained. Three biosynthetic gene clusters, specifically monacolin K, citrinin, and azaphilone pigments that are involved in secondary metabolism, were analyzed and compared. The grouping of strains according to the two-clade groups, designated as (i) *M. pilosu*s and (ii) *M. purpureus*, may play an important role in the food industry and industrial field through the improved utilization of *Monascus* species. The PKS genes responsible for the biosynthesis of azaphilone pigment from the three species were compared. This genome-based analysis showed *M. ruber* could not be clearly grouped as a species. However, in view of food safety, further studies are needed to confirm whether the toxins produced by some *Monascus* strains originate from the genome and not from the metabolites.

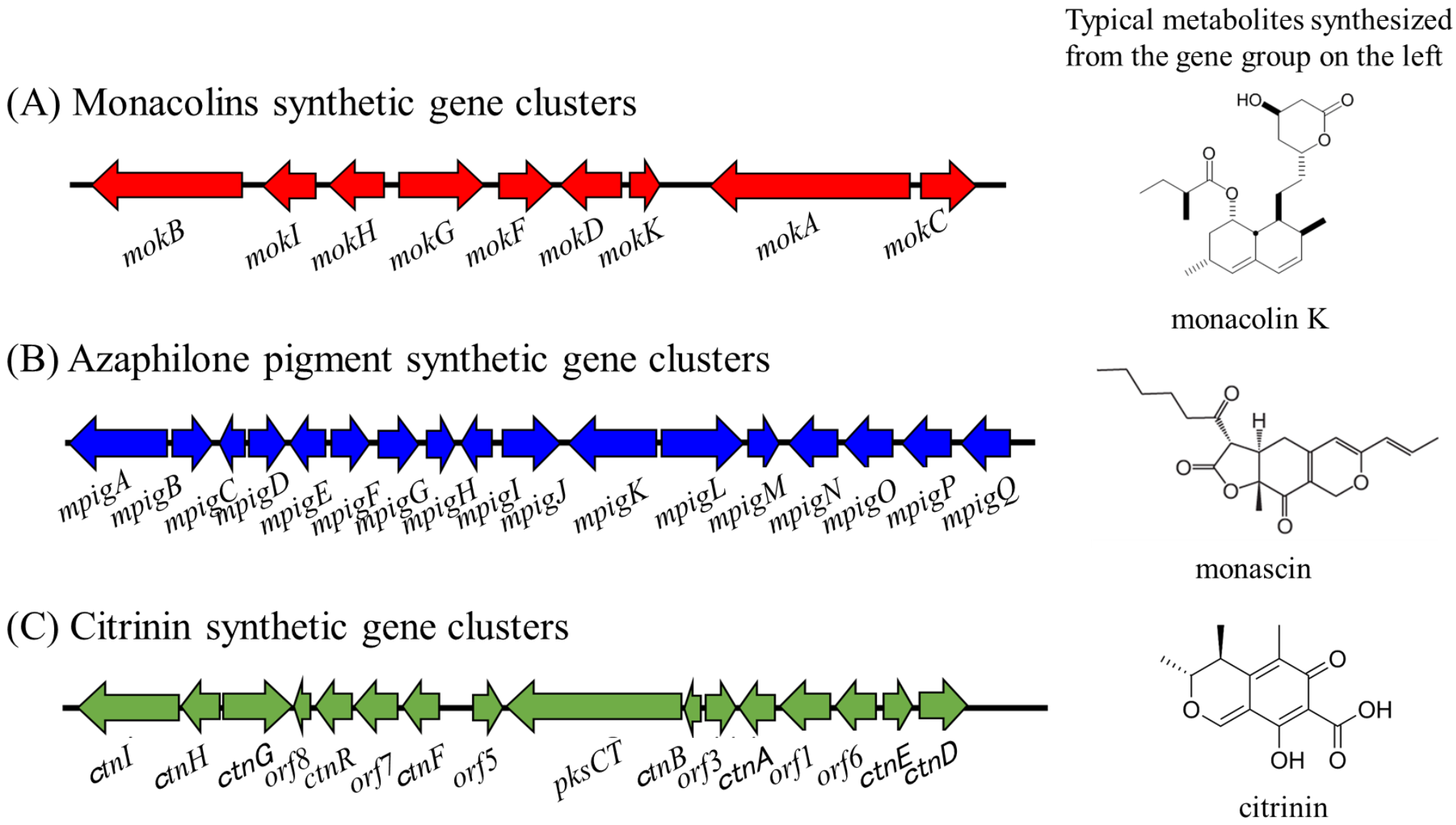


Figure 16 Genetic organization of the monacolins (A), azaphilone pigment (B), citrinin (C) biosynthetic gene clusters.

Table 5 Homologous nucleotides sequences of the citrinin biosynthetic genes in three *Monascus* species.

Protein	*M. pilosus* NBRC 4520		*M. ruber* NBRC 4483		*M. purpureus* NBRC 4478		Reference peptide sequence
	Length	Identity(%)	Length	Identity(%)	Length	Identity(%)	Length
CitA (mpl1)	249	79.6	249	79.6	312	99.7	313
CitB (mpl2)	229	69.6	229	69.6	328	99.7	329
CitD (mpl4)	458	91.4	458	91.4	500	99.8	501
CitE (mpl6)	231	79.1	231	79.1	284	97.3	292
CitC (mpl7)	532	85.5	532	85.5	618	99.4	622
CitS (pksCT)	1525	58.8	1525	58.8	2396	92.4	2593

Table 6 Homologous peptide sequences of the monacolin biosynthetic genes in three *Monascus* species.

Protein	*M. pilosus* NBRC 4520		*M. ruber* NBRC 4483		*M. purpureus* NBRC 4478		Reference peptide sequence
	Length	Identity(%)	Length	Identity(%)	Length	Identity(%)	Length
MokA	1910	100	1910	100	1772	92.8	1910
MokB	1947	100	1947	100	1501	77.1	1947
MokC	339	100	339	100	28	8.3	339
MokD	263	100	263	100	154	58.6	263
MokE	265	100	265	100	231	87.2	265
MokF	258	100	258	100	167	64.7	258
MokG	996	99.0	977	100	322	32.3	977
MokH	487	100	487	100	42	8.6	487
MokI	174	100	174	100	107	61.5	174

Table 7 Comparison of six *M. purpureus, M. ruber,* and *M. pilosus* strains based on Monascus azaphilone pigment polyketide synthase gene

	30	57	88	108	117	123	161	216	219	288	291	321	369	376	392	394	423	442	447	466	471	478	483	516	578	589	606	627	648	663	685	703	748	774	802	820	834	860	939	966	1022	1074	1085	1112	1152	1172	1180	1186	1188	1193
M .pilosus NBRC4520	A	A	A	T	T	G	G	G	A	T	T	A	G	C	G	A	T	A	A	A	A	A	T	C	C	T	A	A	C	T	A	A	T	T	T	C	C	T	T	A	A	T	C	A	A	T	A	C	T	T
M .pilosus BCRC 387072	A	A	A	T	T	G	G	G	A	T	T	A	G	C	G	A	T	A	A	A	A	A	T	C	C	T	A	A	C	T	A	A	T	T	T	C	C	T	T	A	A	T	C	A	A	T	A	C	T	T
M. ruber NRRP 1596	A	A	A	T	T	G	G	G	A	T	T	A	G	C	G	A	T	A	A	A	A	A	T	C	C	T	A	A	C	T	A	A	T	T	T	C	C	T	T	A	A	T	C	A	A	T	A	C	T	T
M. ruber NBRC4483	A	A	A	T	T	G	G	G	A	T	T	A	G	C	G	A	T	A	A	A	A	A	T	C	C	T	A	A	C	T	A	A	T	T	T	C	C	T	T	A	A	T	C	A	A	T	A	C	T	T
M. ruber JF83291.6	C	G	G	C	C	C	A	A	G	C	C	G	A	G	A	G	C	G	G	C	G	G	C	T	T	G	G	G	T	C	G	G	C	G	C	C	A	C	C	G	G	C	T	G	G	C	G	T	C	C
M. purpureus NRRP 1596	C	G	G	C	C	C	A	A	G	C	C	G	A	G	A	G	C	G	G	C	G	G	C	T	T	G	G	G	T	C	G	G	C	G	C	A	A	C	C	G	G	C	T	G	G	C	G	T	C	C
M. purpureus NBRC4478	C	G	G	C	C	C	A	A	G	C	C	G	A	G	A	G	C	G	G	C	G	G	C	T	T	G	G	G	T	C	G	G	C	G	C	A	A	C	C	G	G	C	T	G	G	C	G	T	C	C

	1204	1209	1235	1236	1241	1244	1274	1297	1305	1307	1318	1349	1352	1371	1376	1385	1391	1394	1395	1432	1487	1508	1517	1541	1601	1631	1658	1742	1793	1827	1865	1937	1973	1981	2030	2141	2187	2202	2219	2267	2273	2291	2375	2378	2405	2465	2483	2510	2532	2655
M .pilosus NBRC4520	T	A	G	A	T	T	T	A	A	G	A	A	G	G	T	T	A	T	A	T	C	G	C	C	T	T	A	T	T	C	T	A	A	A	T	A	G	T	G	T	A	A	G	C	T	T	T	G	C	T
M .pilosus BCRC 387072	T	A	G	A	T	T	T	A	A	G	A	A	G	G	T	T	A	T	A	T	C	G	C	C	T	T	A	T	T	C	T	A	A	A	T	A	G	T	G	T	A	A	G	C	T	T	T	G	C	T
M. ruber NRRP 1596	T	A	G	A	T	T	T	A	A	G	A	A	G	G	T	T	A	T	A	T	C	G	C	C	T	T	A	T	T	C	T	A	A	A	T	A	G	T	G	T	A	A	G	C	T	T	T	G	C	T
M. ruber NBRC4483	T	A	G	A	T	T	T	A	A	G	A	A	G	G	T	T	A	T	A	T	C	G	C	C	T	T	A	T	T	C	T	A	A	A	T	A	G	T	G	T	A	A	G	C	T	T	T	G	C	T
M. ruber JF83291.6	C	G	C	G	C	C	G	G	G	C	G	G	A	A	C	C	G	A	C	C	T	A	T	T	C	G	G	C	C	A	C	T	G	G	C	C	A	C	A	G	G	G	A	T	G	C	C	C	T	G
M. purpureus NRRP 1596	C	G	C	G	C	C	G	G	G	C	G	G	A	A	C	C	G	A	C	C	T	A	T	T	C	G	G	C	C	A	C	T	G	G	C	C	A	C	A	G	G	G	A	T	G	C	C	C	T	G
M. purpureus NBRC4478	C	G	C	G	C	C	G	G	G	C	G	G	A	A	C	C	G	A	C	C	T	A	T	T	C	G	G	C	C	A	C	T	G	G	C	C	A	C	A	G	G	G	A	T	G	C	C	C	T	G

Table 7 (continued)

	3919	3936	3953	4025	4058	4088	4151	4169	4248	4310	4373	4425	4481	4484	4592	4660	4670	4673	4775	4785	4808	4912	4927	4937	4958	4974	4985	4987	5021	5035	5036	5182	5194	5198	5230	5234	5276	5281	5291	5292	5293	5294	5301	5302	5363	5417	5442	5448	5471	5489
M .pilosus NBRC4520	T	A	A	C	T	C	A	A	T	G	T	A	A	T	A	T	C	T	A	A	T	T	G	T	A	A	T	C	C	C	C	T	G	A	G	T	T	T	T	-	-	-	G	G	C	C	T	A	A	A
M .pilosus BCRC 387072	T	A	A	C	T	C	A	A	T	G	T	A	A	T	A	T	C	T	A	A	T	T	G	T	A	A	T	C	C	C	C	T	G	A	G	T	T	T	T	-	-	-	G	G	C	C	T	A	A	A
M. ruber NRRP 1596	T	A	A	C	T	C	A	A	T	G	T	A	A	T	A	T	C	T	A	A	T	T	G	T	A	A	T	C	C	C	C	T	G	A	G	T	T	T	T	-	-	-	G	G	C	C	T	A	A	A
M. ruber NBRC4483	T	A	A	C	T	C	A	A	T	G	T	A	A	T	A	T	C	T	A	A	T	T	G	T	A	A	T	C	C	C	C	T	A	A	G	T	T	T	T	-	-	-	G	G	C	C	T	A	A	A
M. ruber JF83291.6	C	G	G	T	C	G	G	T	C	C	C	T	G	C	T	C	T	C	C	C	C	C	A	G	G	G	C	T	T	T	T	G	G	G	A	C	C	G	A	G	A	G	A	A	T	T	G	G	G	G
M. purpureus NRRP 1596	C	G	G	T	C	G	G	T	C	C	C	T	G	C	T	C	T	C	C	C	C	C	A	G	G	G	C	T	T	T	T	G	G	G	A	C	C	G	A	G	A	G	A	A	T	T	G	G	G	G
M. purpureus NBRC4478	C	G	G	T	C	G	G	T	C	C	C	T	G	C	T	C	T	C	C	C	C	C	A	G	G	G	C	T	T	T	T	G	G	G	A	C	C	G	A	G	A	G	A	A	T	T	G	G	G	G

	5492	5495	5500	5501	5527	5552	5568	5599	5615	5618	5634	5674	5714	5726	5780	5795	5807	5819	5882	5906	5987	6021	6028	6041	6149	6167	6173	6200	5021	6236	6248	6329	6347	6383	6389	6521	6569	6572	6596	6641	6759	6914	6928	7002	7006	7007	7008	7019	7024	7082
M .pilosus NBRC4520	C	A	T	A	C	T	A	T	C	C	G	G	C	A	T	C	C	A	G	T	A	T	T	C	T	A	T	A	C	T	T	T	A	A	T	C	C	G	T	G	T	T	T	A	C	A	T	T	G	C
M .pilosus BCRC 387072	C	A	T	A	C	T	A	T	C	C	G	G	C	A	T	C	C	A	G	T	A	T	T	C	T	A	T	A	C	T	T	T	A	A	T	C	C	G	T	G	T	T	T	A	C	A	T	T	G	C
M. ruber NRRP 1596	C	A	T	A	C	T	A	T	C	C	G	G	C	A	T	C	C	A	G	T	A	T	T	C	T	A	T	A	C	T	T	T	A	A	T	C	C	G	T	G	T	T	T	A	C	A	T	T	G	C
M. ruber NBRC4483	C	A	T	A	C	T	A	T	C	C	G	G	C	A	T	C	C	A	G	T	A	T	T	C	T	A	T	A	C	T	T	T	A	A	T	C	C	G	T	G	T	T	T	A	C	A	T	T	G	C
M. ruber JF83291.6	T	G	G	G	T	C	G	C	G	T	A	C	G	G	A	T	T	G	A	G	G	C	C	T	A	G	C	G	T	C	C	C	C	G	C	T	T	C	C	C	C	C	C	G	-	-	-	C	A	T
M. purpureus NRRP 1596	T	G	G	G	T	C	G	C	G	T	A	C	G	G	A	T	T	G	A	G	G	C	C	T	A	G	C	G	T	C	C	C	C	G	C	T	T	C	C	C	C	C	C	G	-	-	-	C	A	T
M. purpureus NBRC4478	T	G	G	G	T	C	G	C	G	T	A	C	G	G	A	T	T	G	A	G	G	C	C	T	A	G	C	G	T	C	C	C	C	G	C	T	T	C	C	C	C	C	C	G	-	-	-	C	A	T

Table 7 (continued)

	7189	7191	7241	7270	7316	7325	7343	7397	7415	7421	7445	7457	7556	7583	7631	7676	7692	7769	7853	7874	7976	8043	8044	8083	8105	8107
M .pilosus NBRC4520	A	A	G	A	C	T	C	A	T	C	T	A	T	A	G	G	A	C	C	T	T	G	T	T	A	A
M .pilosus BCRC 387072	A	A	G	A	C	T	C	A	T	C	T	A	T	A	G	G	A	C	C	T	T	G	T	T	A	A
M. ruber NRRP 1596	A	A	G	A	C	T	C	A	T	C	T	A	T	A	G	G	A	C	C	T	T	G	T	T	A	A
*M. rube*r NBRC4483	A	A	G	A	C	T	C	A	T	C	T	A	T	A	G	G	A	C	C	T	T	G	T	T	A	A
M. ruber JF83291.6	G	C	C	G	T	C	T	C	C	T	C	G	C	G	A	A	G	T	G	C	G	C	C	G	G	G
M. purpureus NRRP 1596	G	C	C	G	T	C	T	C	C	T	C	G	C	G	A	A	G	T	G	C	G	C	C	G	G	G
M. purpureus NBRC4478	G	C	C	G	T	C	T	C	C	T	C	G	C	G	A	A	G	T	G	C	G	C	C	G	G	G

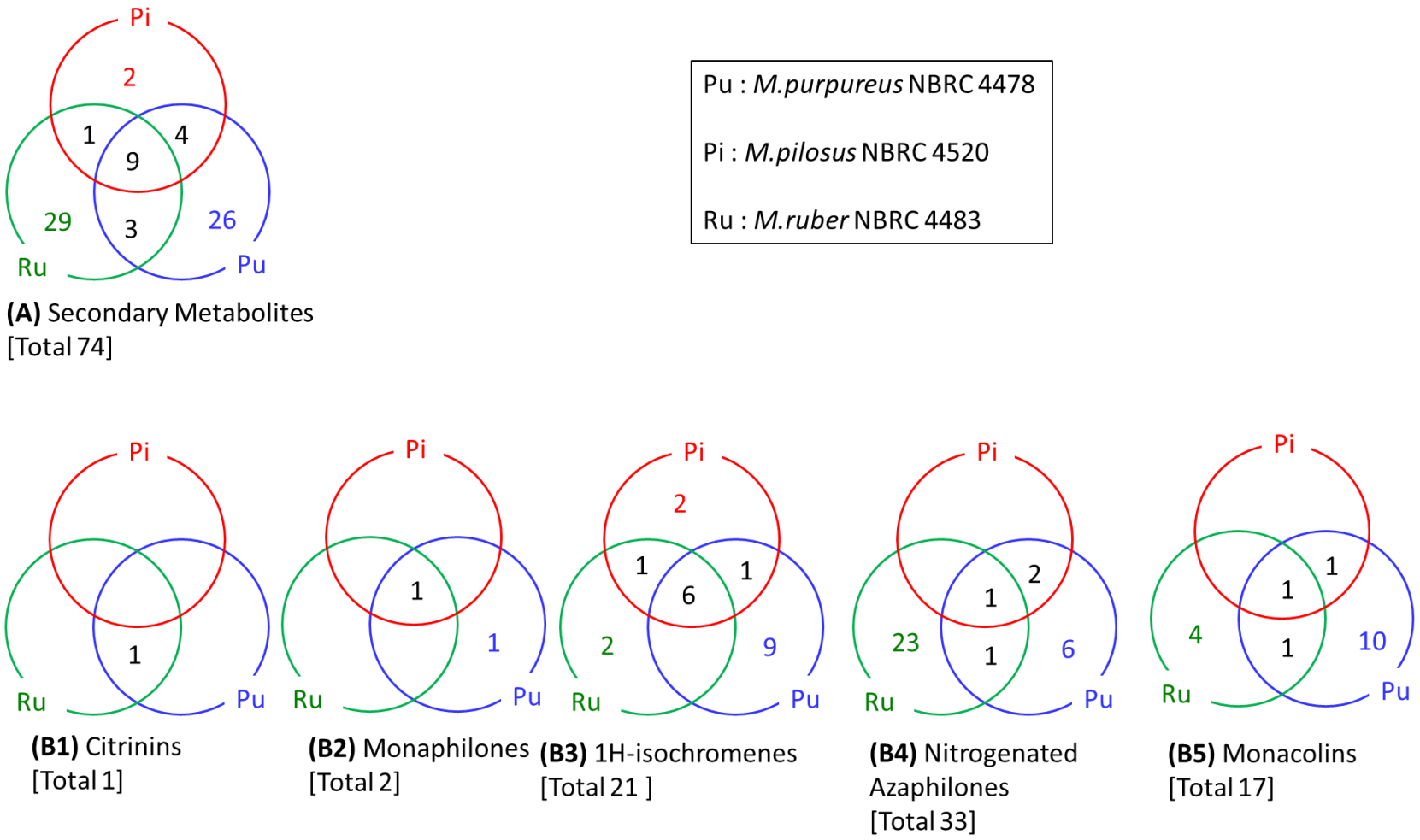


Figure 17 Venn diagrams of *Monascus*-specific metabolites reported by previous studies. Each Venn diagram classifies metabolies into reported species using a total of 74 previously reported secondary metabolites (A),citrinins (B1),monaphilones (B2),1H-isochromenes (B3),nitrogenated azaphilones (B4),and monacolins (B5), specific to *Monascus*-species [9, 40, 77, 85]

Chapter5: Conclusion

In microbiology and food chemistry, systematically controlling the metabolism of microorganisms and controlling metabolites has been one of the important issues for many years. Among them, since *Monascus* has been reported to have various pharmacological actions, studies on culture conditions, gene mutations, and in recent years, metabolic control using genome-editing technology has been reported. In this study, we attempted the difference in phenotype from the genome and metabolites of three types of *Monascus* through data science and analytical chemistry using LCMS. As a result, the following two became clear. First, the genomic information of *Monascus pilosus* was clarified for the first time. As a result, it became clear that the citrinin gene is not possessed. The most commonly eaten red yeast rice in Japan is fermented using *M. pilosus*, and it is a very important finding that it does not carry the mycotoxin gene. Second, the gene structure and secondary metabolites of each were analyzed, and the characteristics of each *Monascus* species were clarified. As a result, it was clarified that the ORF gene was not significantly different, but the metabolites of each strain were different. It is considered that the gene sequence other than the region encoding the protein has a great influence on the production of secondary metabolites. In this study, the genome and metabolites were analyzed in an integrated manner, and the characteristics of the phenotype of red yeast rice eaten by humans were clarified. It is thought that this can be applied not only to red yeast rice but also to other fermented foods such as natto, yogurt, and tempeh. The fermentation process of microorganisms is very complicated. By integrating genome analysis and metabolome analysis and standardizing phenotypic analysis, fermented foods can contribute to our health more scientifically. The accumulation of these findings will provide data that can contribute to the long-standing task of systematically controlling the metabolism of microorganisms and controlling metabolites.

Research achievement

- The contents of chapter 2.1, 2.2, 2.3, 3.1, 3.2, 3.4 and were publicshed as : Divergence of metabolites in three phylogenetically close Monascus species (*M. pilosus, M. ruber,* and *M. purpureus*) based on secondary metabolite biosynthetic gene clusters. Yuki Higa, Young-Soo Kim, Md. Altaf-Ul-Amin, Ming Huan, Naoaki Ono, Shigehiko Kanaya, *BMC Genomics*, volume 21, Article number: 679 (2020)
- The contents of chapter 2.1, 2.2, 2.3, 3.1, 3.2, 3.4 and were presented as: Food application usefulness of *Monascus pilosus* NBRC4520 revealed by comparative genome analysis of three species of *Monascus*. Yuki Higa, Shigehiko Kanaya, Young-Soo Kim, Naoaki Ono. Japan Society for Bioscience and Biotechnology in 2019 (oral session).
- The contents of chapter 2.1, 2.2, 2.3, 3.1, 3.2, 3.4 and were presented as: Comparative genome analysis of *Monascus* focusing on secondary metabolite genes. Yuki Higa, Young-Soo Kim, Md. Altaf-Ul-Amin, Ming Huan, Naoaki Ono, Shigehiko Kanaya. Japanese Society of Food Chemistry in 2020 (oral session). We received the Young Excellence Award.

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