Divergence of pigments in three phylogenetically close *Monascus* species (*M. pilosus*, *M. ruber,* and *M. purpureus*) based on secondary metabolite biosynthetic gene clusters

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Abstract

Background: Species under the genus Monascus are considered as economically important and have been widely used in the production of yellow and red food colorants. In particular, three Monascus species, namely, M. pilosus , M. purpureus , and M. ruber , are used for food fermentation in the cuisine of East Asian countries such as China, Japan, and Korea. These species have also been utilized in the production of various kinds of natural pigments.

Results: We examined the diversity of pigment-related biosynthetic pathways in three Monascus species (M. pilosus , M. purpureus , and M. ruber) at the metabolome and genome levels. Illumina MiSeq 300 bp paired-end sequencing generated 17 million high-quality short reads in each species, corresponding to 200 times the genome size. We measured the pigments and their related metabolites using potato dextrose liquid (PDL) media. The colors in the PDL media corresponding to the pigments and their related metabolites produced by the three species are very different from each other. The gene clusters for secondary metabolite biosynthesis of the three Monascus species also diverged, confirming that M. pilosus and M. purpureus are chemotaxonomically different. M. ruber has similar biosynthetic gene clusters for citrinin, monacolin K, and Monascus azaphilone pigments with M. pilosus and M. purpureus. The comparison of secondary metabolites produced also revealed divergence in the three species.

Conclusions: Our findings are important for improving the utilization of Monascus species in the food industry and industrial field. However, in view of food safety, we need to determine if the toxins produced by some Monascus strains exist in the genome or in the metabolome.

Background

Species of the genus Monascus are economically important because they have been widely used in the production of yellow and red food colorants. In particular, M. pilosus, M. purpureus, and M. ruber are commonly used for food fermentation in the cuisine of East Asian countries including China, Japan, and Korea (Wang and Lin, 2007; Lee and Pan, 2012; Yasuda et al., 2012) and utilized to produce various kinds of natural pigments(reviewed in Gao et al., 2013; Eman et al., 2014), such as yellow pigments (ankaflavin, monascin, rubropunctatin), orange pigments (monascorubrin), purple pigments rubropunctamin, monascorubramin), and red pigments monascorubramine, N-glucosylmonascorubramine, N-glucosylrubropunctamine, N-glutarylmonascorubramine, N-glutarylrubropunctamin). One example is the traditionally fermented rice which contains at least 6 pigments from Monascus spp., including rubropunctatin, monascorbrin, rubropunctamin, monascorubramin, ankaflavin, and monascin (Pastrana et al., 1995).

M. pilosus is a well-known fungus that produces several bioactive metabolites, such as monacolins K and L, as well as several pigments that are related with biological activities including anti-obesity, regulation of lipid metabolism, and Alzheimer’s disease at the in vitro and in vivo levels (Agboyibor et al., 2018).

The complete genome sequence of the industrial strain M. purpureus YY-1 is already available (Yang et al., 2015). However, the genome sequences of M. ruber and M. pilosus are still incomplete. Understanding the diversity of the pigments produced by these species at the genome level is remarkably important for their industrial applications. We analyzed M. pilosus, M. purpureus, and M. ruber to determine the diversity of the pigments based on metabolome data and pigment-related gene clusters. Several pigments are synthesized through the PKS and NPRS systems responsible for organizing gene clusters in the genome. Comparison of gene clusters between the three species will provide new insights to the potential production of novel pigments.

Monascus species produce a multitude of compounds, including polyketides, unsaturated fatty acids, phytosterols, pigments, and monacolins. Monacolins, especially monacolin K, inhibit 3-hydroxy-3-methylglutaryl-coenzyme A reductase, which is the rate-limiting step in cholesterol biosynthesis. These compounds found in red yeast rice prevent the production of high cholesterol level that causes atherosclerosis (Gerards et al., 2015). Hence, it is expected that metabolites related with Monascus pigments can contribute to human healthcare. However, citrinin was found as an undesirable contaminant in red yeast rice (Fink-Gremmels et al., 1991).

Thus, it is required to clarify the diversity of pigment biosynthetic pathways in economically important Monascus species. In the present study, we determined the genome sequences of M. pilosus, M. purpureus, and M. ruber. The phylogenetic and chemotaxonomic differences between the three were characterized by analyzing the gene clusters of secondary metabolites. The pigment production in M. pilosus was also further characterized.

Results

Production of secondary metabolites

Monascus species can produce a several types of azaphilones, including 1H-isochromenes, nitrogenated azaphilones, citrinins, and monacolins (Gao et al., 2013). We measured the pigments and their related metabolites in three Monascus species using potato dextrose liquid (PDL) media, which is the frequently used and suitable culture media for Monascus growth and metabolite production (Carvalho et al., 2003). As shown in Fig. 1a, the colors in the media cultured with the individual species are distinct. A gradient of orange to light yellow (from center toward edges) was observed in M. pilosus, gray to light yellow in M. ruber, and pink in M. purpureus. We also observed the gradients from light orange in M. pilosus, from pale orange to black color in M. ruber, and from red to dark purple in M. purpureus. Thus, the colors were reflected by the different pigments produced by the three species cultured in identical conditions. We also analyzed the pigment-related metabolites using LC-MS and identified 14 metabolites (Fig. 1b). The reproducibility of metabolite quantities was confirmed by three iterative measurements. Dehydromonacolin K, rubropunctatin, monascin, and ankaflavin 2 were commonly produced by the three species. M. pilosus produced 12 metabolites, which is higher than the others (Fig. 1c). 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 (Nejati et al., 2014), was only produced by M. purpureus.

The biosynthetic pathway from malonyl-CoA to1H-isochromenes was determined in Penicillium marneffei and M. ruber (Woo et al., 2014; Chen et al., 2017). Citrinin polyketide synthase (PKS) converts the PKS-bound product citrinin (He and Cox, 2016). The biosynthetic pathway from malonyl-CoA to monacolins was also determined in Aspergillus terreus, M. ruber, and M. purpureus (Cambell et al., 2010; Zhang et al., 2017). 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 (Chen et al., 2017) and the other is citrinin polyketide synthase which corresponds to the pathway from PKS-bound product to citrinin (He and Cox et al., 2016). Figure 2 shows 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 levels of metabolites accumulated based on 2D clustering results in Fig. 1b. Among the three Monascus species, some of the metabolites related with 1H-isochromenes were accumulated through the biosynthetic pathways from malonyl-CoA to 1H-isochromenes. All eight metabolites related with 1H-isochromenes were only detected in M. pilosus. Pigments produced by M. pilosus are more suitable for observation in PDL medium than the other two species. Citrinin was only observed in M. purpureus. Dehydromonacolin K, which is a precursor for monacolin K production, was detected in all three species.

Thus, the accumulation of pigment-related metabolites may be different among the three Monascus species, as observed using identical culture conditions in PDL medium. Metabolites related with 1H-isochromenes and nitrogenated azaphilones were observed among the three, but the metabolites were different between individual species. On the other hand, metabolites related with ankaflavin 1 and rubropunctatin were observed in M. pilosus, while monascorubramine was detected in the other two.

Discussion

The three Monascus species examined in the present study are commonly used for food fermentation in the cuisine of East Asian countries (Wang and Liu, 2007; Lee and Pan, 2012; Yasuda et al., 2012). Citrinin, is a nephrotoxic agent, was reportedly produced in M. purpureus but not in M. pilosus (Wong et al., 1977; Ma et al., 2000; Rasheva et al., 2003). This is corroborated by the present results from the metabolome and genome analyses revealing that the biosynthetic genes in M. pilosus were insufficient compared with those from M. purpureus. The three Monascus species can produce ubiquitous and species-specific pigment-related compounds (Figs. 2 and 3). The gene-organization revealed the greatly diverged 54 gene clusters in the three Monascus species studied (Fig. 5a2). Furthermore, comparison of a 8,144 bp region in which a biosynthetic gene cluster of Monascus azaphilone pigment 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 2).

M. pilosus is treated as a synonym of M. ruber in the concatenated phylogeny based on the ITS, BenA, CaM LSU and RPB2 gene regions (Barbosa et al., 2017) and the partial beta-tubulin gene (Park et al., 2004). These genes are associated with highly conserved in the genomes of even distantly related species. However, 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 can be classified into two groups: (i) the M. pilosus clade and (ii) the M. purpureus clade. M. ruber strains can be grouped with any of the two clades.

The mycotoxin citrinin is produced by various Penicillium, Aspergillus, and Monascus species (Wong et al., 1977; Ma et al., 2000; Rasheva et al., 2003). Previously studied M. purpureus strains (ATCC 16365 in Java, 16379 in Taiwan, IFO 30873, and DSM 1379 by Chen et al., 2008; Ostry et al., 2013; YY1 by Liang et al., 2018) can produce citrinin. However, among the Monascus species, two M. pilosus strains (BCRC 38072 in Taiwan by Chen et al., 2008; 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 et al. (2008), AUMC 4066 (CBS109.07) and AUMC 5705 by Moharram et al. (2012), NRRP 1597 by Kwon (2016), and NBRC 4483 in this study, lack citrinin production activities, but other strains, such as Tiegh by Ostry et al. (2013) and ATCC 96218 by Hajjaj et al. (1999) have the potential to produce citrinin. 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 monacolin K gene cluster, four M. purpureus strains, specifically NRRP 1596, YY-1, KACC 42430 (Kwon et al., 2016), 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 the primary metabolites that are essential for growth and reproduction and the 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. Citrinin pathway belongs to the former as it is present in many Penicillium, Aspergillus, and Monascus species (Wong et al., 1977; Ma et al., 2000; Rasheva et al., 2003). 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 (Floudas et al., 2012). A second source of metabolic gene innovation in fungi is horizontal gene transfer (HGT), which includes xenobiotic catabolism (Gardiner et al., 2012), toxin production (Friesen et al., 2006), and degradation of plant cell walls (Garcia-Vallve et al., 2000). GD and HGT were more frequently occurring in clustered genes than in their non-clustered counter parts (Wisecaver et al., 2014). 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 trends. 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 pigments, is the most useful role for the divergence in the Monascus genera.

Conclusions

In this study, the complete 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 azaphilon pigments that are involved in secondary metabolism, were analyzed and compared. 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, further studies are needed to confirm whether the toxins produced by some Monascus strains originate from the genome and not from the metabolome.

Methods

Strains, culture condition, and metabolite detection

Three *Monascus* species, specifically, *M. pilosus* NBRC 4520, *M. purpureus* NBRC 4478, and *M. ruber* NBRC 4483, were obtained from the National Institute of Technology and Evaluation in Japan. The three species were cultured in potato dextrose liquid medium at 30°C for 7 days with 140 rpm shaking in TAITC BR-23FP. A solution of 10 mg freeze-dried PDL medium added with 1 mL methanol was sonicated for 30 min to extract secondary metabolites. The extracted metabolites were measured using Shimadzu LCMS-8040 (Shimadzu, Kyoto, Japan) with 300 mm ODS MonoBis columns (Kyoto Monotech Co., Ltd., Kyoto, Japan).

Genome sequencing and assembly

We isolated genomic DNA from the three species individually and sequenced them using Illumina MiSeq paired-end libraries (0.3 Kb). Approximately 8.5 million reads (around 5 Gb) for each sample were obtained and assembled using ABySS 2.0 *de novo* assembler (Jackman et al., 2017). The assembled

scaffolds had an N50 value of 133 Kb. The total length of the assembled contigs was 24.8 M bp, which is close to that of *M. purpureus* NRRP 1596 genome (ATCC 16365) with 23.4 Mb (Chen et al., 2008) and *M. ruber* NRRP 1597 (ATCC 13692) with 24.9 Mb (Chen et al., 2008). 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 (Buchfink et al., 2015). The assembled sequences were also BLASTed against the UniProtKB/Swiss-Prot database (Pundir et al., 2017) and the genomes of *M. purpureus* NRRP 1596 and *M. ruber* NRRP 1597 for validation, using a cutoff of E-value < 1E-10. We further analyzed the genomes using antiSMASH pipeline (Medema et al., 2011) to extract the functional gene clusters such as PKS, in each *Monascus* species.

Declarations

Ethics approval and consent to participate: Not applicable

Consent for publication: Not applicable

Availability of data and materials: Not applicable

Competing interests: Not applicable

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Authors' contributions

Conceptualization and design of the study were performed by YH, SK, and NO. Sample preparation and genomic DNA isolation were carried out by YH. Assembly and scaffolding of sequencing reads were performed by NO. Subsequent comparative genomic analysis were conducted by NO and YH. Statistical processing and figure creation were conducted by SK. Culture and LC-MS analysis were performed by YH. Valuable comments and advice on writing papers were provided by AA, MK, and YSK. All authors have read and approved the final manuscript.

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Tables

Due to technical limitations, tables are only available as a download in the supplemental files section

Figures

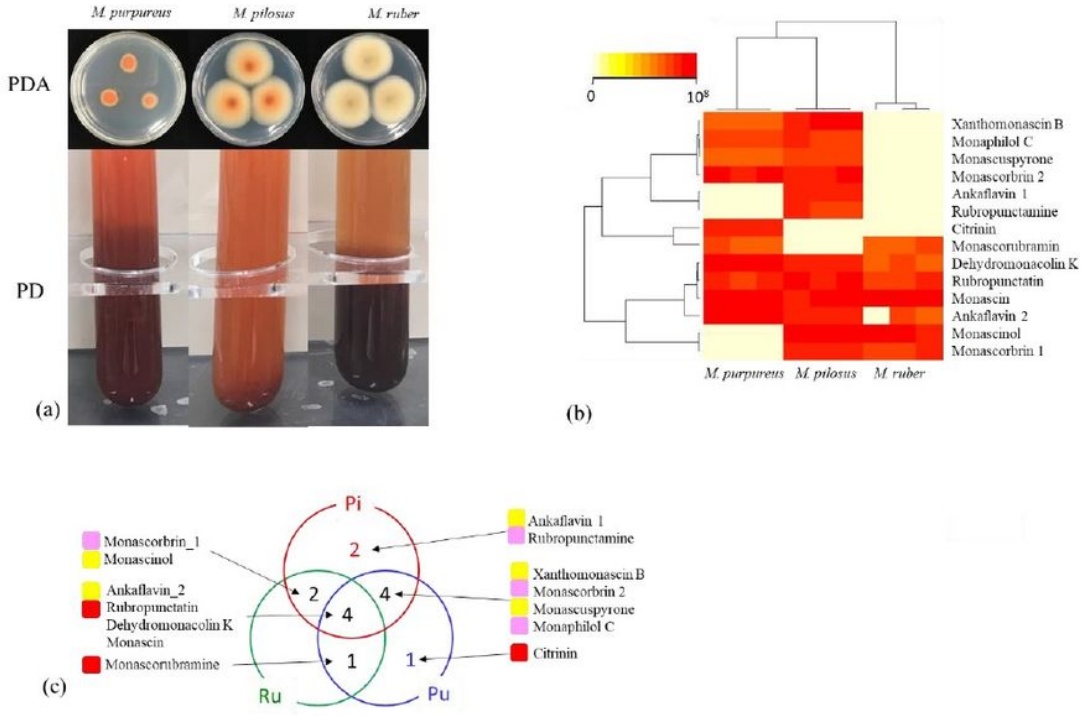

Figure 1

Pigments for three Monascus species for PD agar and PDL cultures (a) Monascus species are cultured in PD agar and PDL medium at 30 °C . (b) 2D clustering of pigment quantities among the three species (c) Venn diagram of the pigments observed in PD L culture.

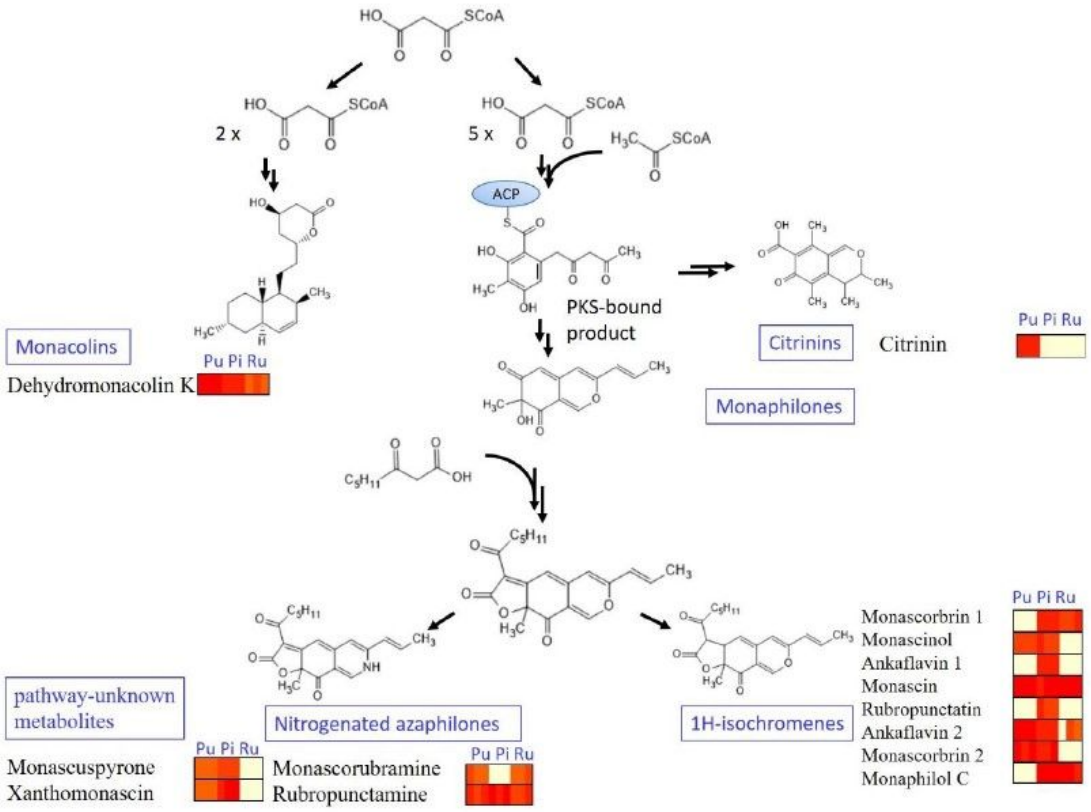

Figure 2

Accumulation of three metabolite clusters corresponding to the pigments observed in Figure 1(b). The red, and blue colors correspond to metabolite c lusters 1, 2, and 3 , respectively Two letter abbreviations use used for the Venn diagrams: Pi, M. pilosus NBRC 4520; Ru, M. ruber NBRC 4483; Pu, M. purpureus NBRC 4478.

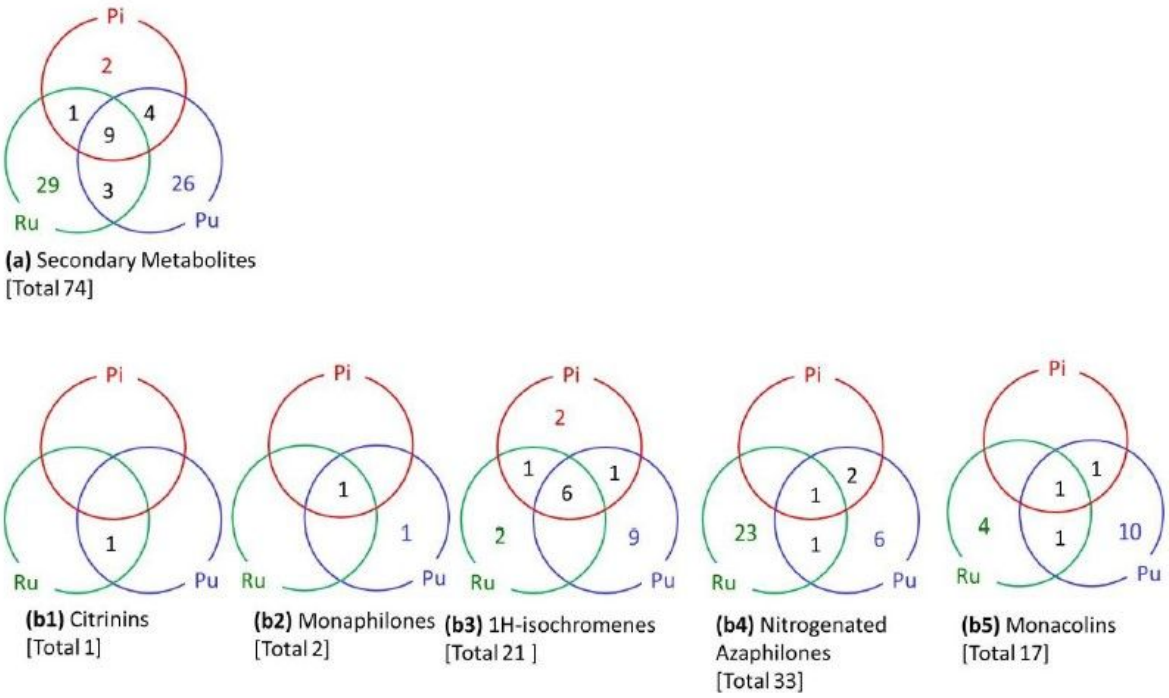

Figure 3

Venn d diagram s of Monascus specific metabolites reported by previous studies 14, 21, 24, 30, 31, 25, and 50 Venn diagram classified into reported species using a total of 74 previously reported secondary m etabolites (a),citrinins (b1), monaphilo nes (b2),1H isochromenes (b3),nitrogenated azaphilones (and monacolins (b5), specific to Monascus species. Two letter abbreviations use used for the Venn diagrams: Pi, M. pilosus NBRC 4520; Ru, M. ruber NBRC 4483; Pu, M. purpureus NBRC 4478

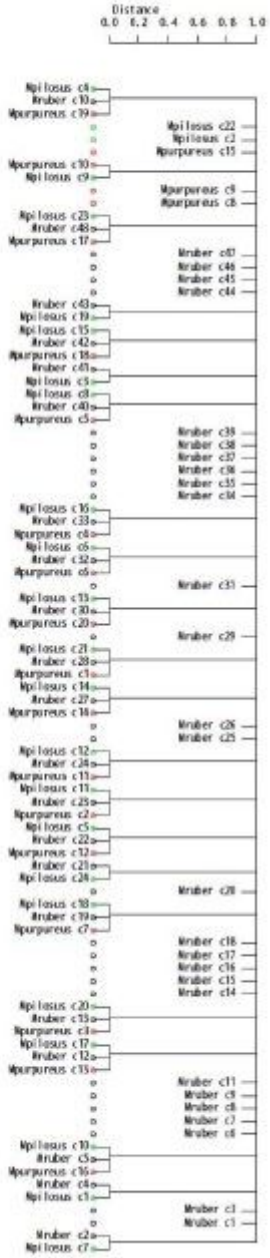

Figure 4

Dendrogram of secondary metabolite biosynthetic gene clusters observed in three Monascus species.

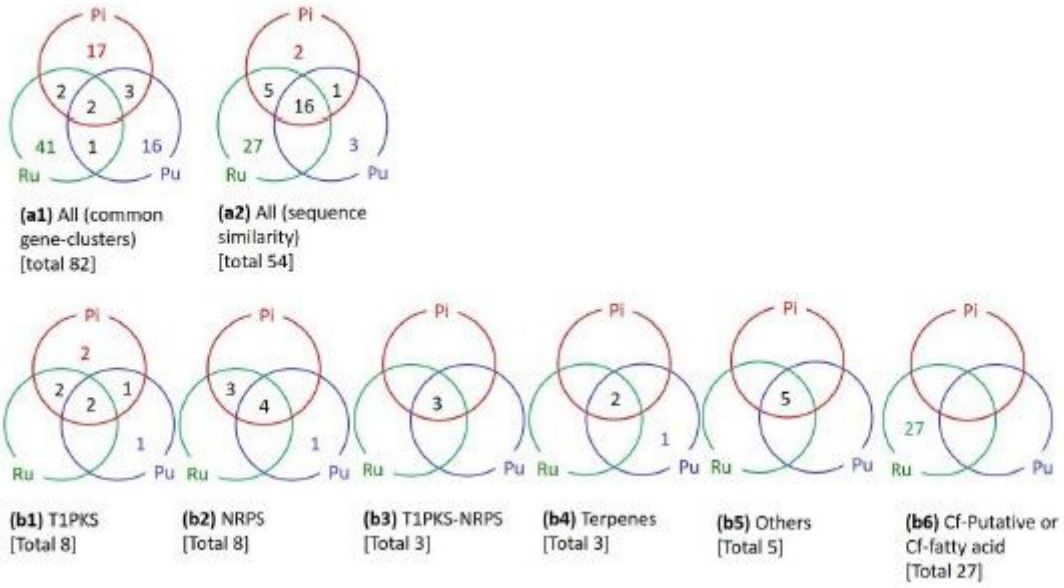

Figure 5

Figure 5 Venn diagram s of secondary metabolite biosynthetic gene clusters observed in three Monascus species (a1) Venn diagram classifying 82 secondary metabolic synthesis genes of three Monascus species. (Venn diagram classifying 82 secondary metabolic synthesis genes of three Monascus species based on DNA sequence homology. The total number of genes was 54. Venn diagram classifying 54 secondary metabolic synthesis genes of three Monascus species based on D NA sequence homology: T1PKS (b1), NRPS (b2)), T1 PKS NRPS (b3)), Terpenes b4)), Others (b5)), Cf Putative or Cf fatty acid (b6). Two letter abbreviations use used for the Venn diagrams: Pi, M. pilosus NBRC 4520; Ru, M. ruber NBRC 4483; Pu, M. purpureus NBRC 4478.

Supplementary Files

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- Tables20200121higa.pdf