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MOLECULAR PHYLOGENETIC ANALYSIS OF *MONASCUS* FUNGI BASED ON INTERNAL TRANSCRIBED SPACER REGION

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ABSTRACT

A molecular analysis of internal transcribed spacer region has been carried out to reveal the relationship among 16 strains of *Monascus* spp. A primer set comprised primer ITS1 and ITS4 was used to amplify this region in which they were cloned and scqucnccd. We also compared the sequence result with *M. purpureus* AF458473, *M.ruber* AF458470, *M. kaoliang* AF451859, *M. araneous* AF458471 and *M. pilosus* AF451856 and one outgroup species *Thermoascus crustaceus* U18353.

The result showed that 16 *Monascus* spp. were divided into two large clades while *M. ruber* AF458470 was basically separated from all those *Monascus.* One of the two large clades included the seven *M. purpureus* strains, *M. purpureus* AF458473, *M. araneosus* AF458471 and *M. kaoliang* AF451859. Another large cladc included the six *Monascus* sp. strains which typically have whitish colonies, the three *M. ruber* strains and *M.pilosus* AF451856.

However, even outstanding morphological differences possessed by several white *Monascus* and one whitish *M. purpureus* strain, all *Monascus* strains were suggested to be very closely related with similarity >99% almost 100%. Although this ITS analysis could not discriminate cultural and morphological differentiation *of Monascus* strains studied, yet there is still little genetic variation within these strains.

Key words : Molecular genetics/Monascus spp./fungi

INTRODUCTION

Hawksworth and Pitt (1983) cited that *Monascus* species (Monascaceae) are important for producing Asian fermented foods particularly red rice (ang-kak), rice wine and kaoliang brandy, soy bean cheese and food colorants; for their antibacterial properties; production of mycotoxin; and a major component of silage mycofloras. Lakrodi *et* al. (200) cited that one of these world wide distributed fungi, *M. purpureus* is known as the red rice fungus that has been used for over a thousand years by the Chinese as a traditional herbal medicine. However, this fungus was firstly isolated from Chinese red rice (ang-kak) in Java.

In Indonesia, we isolated and collected *M. purpureus* from ang-kak in Java and Sumatra and from other several *Monascus* species isolated from deteriorated invertebrate specimens. Based on morphological observation on growth in agar media, we found some interesting not ordinary properties in *M. purpureus* such as two *M. purpureus* isolates one of which has unique character such as bigger ascomata and the other one has white colony. While the other four *Monascus*

isolates have very remarkable properties such as they resist ethanol at very extreme concentration and their morphological characters are also unique. So, it is of interest to know the genetic relationship rather than morphological relationship among those isolates within *Monascus* species. Actually, DNA sequence analysis based on the D1/D2 regions of LSU rRNA genes *of Monascus* species have been conducted by Park and Jong (2003). They suggested that *M. lunispora, M. floridanus,* and M *pallens* were separated in different clades. However, five *Monascus* species such as M *pilosus, M. purpureus, M. ruber, M. eremophilus* and *M. sanguineus* were reflected in monophyletic relationship (Park and Jong 2003). These five species were definitely different species based on morphological characters on agar media by Hawksworth and Pitt (1983) *(M. pilosus, M. purpureus, M. ruber),* Hocking and Pitt (1988) (M *eremophilus)* and Cannon *et al.* (1995) (M *sanguineus).*

As Park and Jong (2003) have suggested no separation among the five species of *Monascus,* we intended to do analysis on internal transcribed spacer region of *Monascus.* This region is known for its various nucleotides sequences so it might be more perspective rather than on LSU rRNA genes. This similar work was not only to reveal genetic diversity of *Monascus* in Indonesia, but also to understand the relationship among *Monascus* species and direction of mutation by analysis

MATERIALS AND METHODS

Monascus Strains

A number of 16 Monascus strains used in this study were isolated and identified by Suharna in 2002 and 2003 (Table 1).

Table 1. *Monascus* strains used

Monascus Strain	Isolation Year	Source of Isolation
1. M. purpureus JMBa	2002	Fermented Chinese red rice
2. M. purpureus JMS	2002	Powdered Medicinal Herb
3. M. purpureus NGK-J	1995	Fermented Chinese red rice
4. M. purpureus PKB1	2003	Fermented Chinese red rice
5. M. purpureus PKB5	2003	Fermented Chinese red rice
6. M. purpureus PRBa	2002	Fermented Chinese red rice
7. M. purpureus SRBa	2002	Fermented Chinese red rice
8. M. ruber CKA1	2003	Acid Liquor of *Arenga pinnata*
9. M. ruber CKA3	2003	Acid Liquor of *Arenga pinnata*
10. M. ruber SKF11	2003	Acid Liquor of *Arenga pinnata*
11. Monascus sp. COEL	1994	Invertebrate Specimen
12. Monascus sp. KA30.1	1994	Invertebrate Specimen
13. Monascus sp. KTB	1994	Invertebrate Specimen
14. Monascus sp. MM	1994	Invertebrate Specimen
15. Monascus sp. MYOM	1994	Invertebrate Specimen
16. Monascus sp. MYOT	1994	Invetebrate Specimen

Cultivation and purification *of Monascus* strains

All fungi were cultivated on YM Agar plate and for purification of cultures Water Agar 2% was used. Incubation was carried out at room temperature (25°C) for three days. A little amount of mycelial mass of each fungus was picked up using toothpick then transferred into centrifuge tube containing 10 ml of YM broth medium and incubated at 30°C for three days. The mycelial mass of each fungus was then harvested for DNA extraction.

DNA extraction

The harvested mycelial mass of each fungus was put onto paper to remove excess of medium then subsequently put onto mortar before freezing by pouring liquid nitrogen and grounded by mortar and pestle. The following step of DNA extraction was carried out using QIAamp tissue kit (QIAGEN). The yield of DNA samples was quantified and qualified by both spectrophotometer and gel electrophoresis.

PCR, cloning and sequencing of ITS region

ITS region was amplified by PCR with specific primer set, ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') (White *et al.* 1990). PCR was conducted using *AmpliTaq* DNA polymerase (Roche, Basel, Switzerland) and supplemented with buffer system under a temperature profile of 94 °C for 4 min. followed by 30 cycles of 94 °C for 30 sec, 55 °C for 1 min. and 72 °C for 1 min. The PCR products were directly cloned with TA-cloning vector pT7Blue (Takara) and *Escherichia coli* DH5a competent cells (Takara) using ampicillin and X-gal blue-white selection system. To check the length of the inserted DNA fragment, white colonies expected to contain inserted plasmid were directly subjected to PCR using the primers Univl9 (5'-GTTTTCCCAGTCACGACGT-3') and Rev20(5'-AGCTATGACCATGATTACGC -3'). When a PCR product of expected size (600 bp) was obtained, two clones of which were cultured overnight in 3 ml of LB medium containing ampicillin, and subjected to plasmid extraction using QIAprep-Spin Miniprep Kit (QIAGEN). The purified plasmids were eluted with 50 ul distilled-water and used for sequencing. Dye terminator-labelled cycle sequencing reaction was conducted with DNA Sequencing Kit FS (Perkin Elmer) and four sequencing primers Univl9 and Rev20 under a temperature profile of 94 °C for 4 min. followed by 30 cycles of 94 °C for 30 sec, 50 °C for 1 min. and 60 °C for 4 min. The products were analyzed by ABI PRISM 377 DNA sequencer (Perkin Elmer).

Molecular phylogenetic analysis

The ITS region sequences determined were subjected to molecular phylogenetic analysis together with those retrieved from the DDBJ nucleotide sequence database. A multiple alignment of the ITS region sequences was generated by the program package Clustal W (Thompson *et al.* 1994). Phylogenetic trees were constructed by the neighbor-joining method using Clustal W (Thompson *et al.* 1994). Bootstrap tests (Felsenstein 1981) were performed with 1000 replications.

RESULTS

Figure 1 shows that amplification by primer set comprised ITS1 and ITS4 were successful for all *Monascus* strain tested.

The size of amplified DNA products of all *Monascus* isolates by the primer set was 700 base pair each as shown in Figure 1.

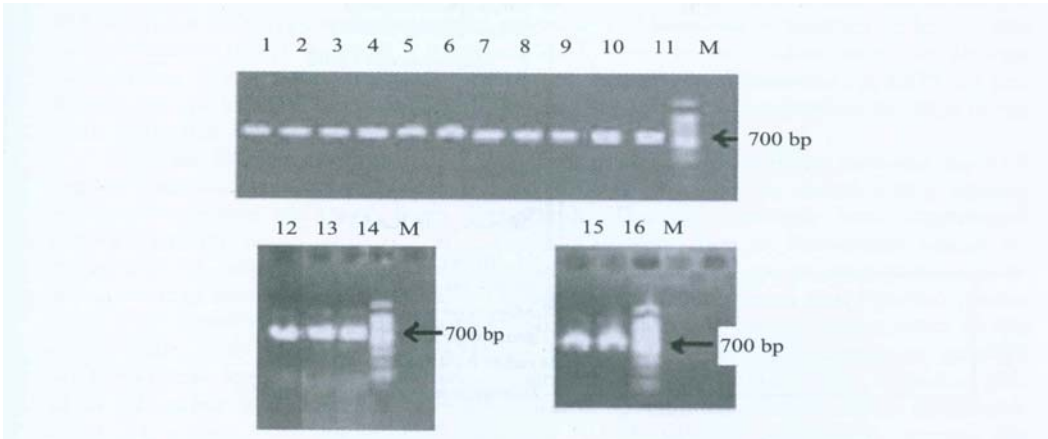

Figure 1. Amplified DNA product of 16 *Monascus* strains by a primer set comprised primer ITS1 and ITS4. Line: 1. PKB1, 2. JMBa, 3.SKF11, 4.COEL, 5. MM94, 6.JMS, 7.SRBa, 8.KTB, 9.MYOT, 10. MYOM, 11. NGK-J 12.KA30.1, 13.CKA3, 14. CKA1, 15. PKB5, 16. PRBa.

Molecular phytogeny

Figure 2 shows that *Thermoascus crustaceus* U18353 used as outgroup was significantly separated from all *Monascus* species that integrated in one main clade. This *Monascus* clade consists of very similar two large clades, while *M. ruber* AF458470 was basically separated from all those *Monascus.* One of the two large

clades includes the seven *M. purpureus* strains, *M. purpureus* AF458473, A *araneosus* AF458471 and *M. kaoliang* AF451859. Another large clade includes th six *Monascus* sp. strains which typically have whitish colonies, the three *M. rube* strains and *M.pilosus* AF451856. The similarities within the seven *M. purpurei>* strains and *M. purpureus* AF458473, *M. araneosus* AF458471 and *M. kaolian* AF451859 are very high more than 99% or almost 100%. However, *M. purpure^* PKB5 shows a little more different. The six *Monascus* sp. strains, the three *M. rube* and *M. pilosus* AF451856 also showed very high similarity. But lesser difference i shown by *Monascus* sp. KA30.1.

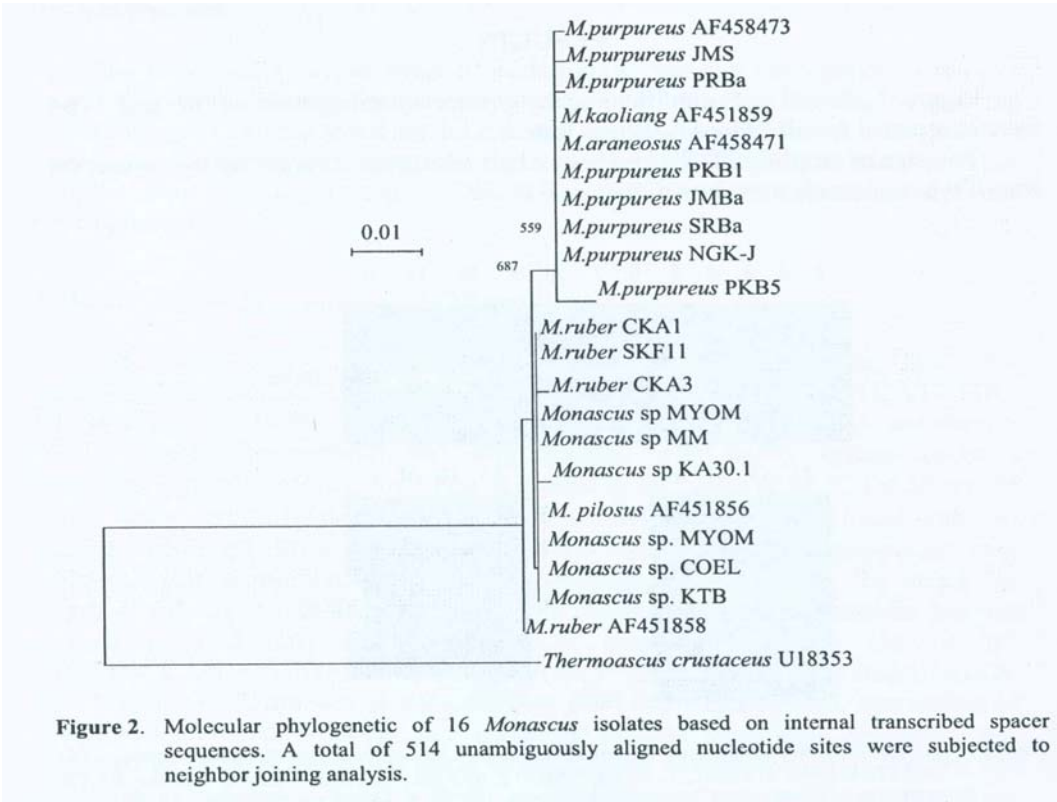

Figure 2. Molecular phylogenetic of 16 *Monascus* isolates based on internal transcribed spacer sequences. A total of 514 unambiguously aligned nucleotide sites were subjected to neighbor joining analysis.

DISCUSSIONS

With regard to the level of very high similarity more than 99% almost nearl; 100% among all *Monascus* fungi we analyzed, indicated that there is little mutatioi on ITS region of *Monascus* species. Concerning genetic diversity it seemed tha there is a diverse mutation tendency as several *Monascus* strains such a

M.purpureus PKB1, *M.purpureus* PRBa, *M.purpureus* JMS, *M.purpureus* AF458473, *M. rubber* CKA1 and *Monascus* sp. KA30.1 appeared more different though these differences were so slight. Therefore, this phylogenetic analysis revealed very close relationship among *Monascus* strains so we suggested that all *M.purpureus* were identical to each other as shown by *M.purpureus* NGK-J, *M.purpureus* JMBa, *M.purpureus* PKB1, *M.purpureus* PKB5, *M.purpureus* SRBa, *M.purpureus* AF458473 and *M. araneosus* AF458471 in the same clade. In another clade, all Indonesian *M. ruber* strains and *M. pilosus* were identical to each other. However, *M. ruber* AF458470 was separated in another clade. This work also confirmed that *M. araneosus* AF458471 and *M. kaoliang* AF451859 are the same species with *M. purpureus*. Previously, Hawkswoth and Pitt (1983) have included *M. araneosus* and *M. kaoliang* as *M. purpureus* synonym based on morphological characteristics. It is also of interest to note that *M. purpureus* SRBa has a uniqueness of its morphological features such as having bigger ascomata (generally 90 um in diameter) than "normal" *M. purpureus.* The normal *M. purpureus* has ascomata up to 70 um in diameter. The other white isolates such as *Monascus* sp. MM, *Monascus* sp. COEL, *Monascus* sp. KTB, *Monascus* sp. MYOM and *Monascus* sp. MYOT have different morphological characters. These white isolates at least have two different morphological characters compared to *M. ruber* such as shorter ascospores, cleisthothecium wall transparent. Moreover, *Monascus* sp. MYOM and *Monascus* sp. MYOT have fusiform aleurispores, while *M. ruber* has no this spore form(Suharna 1999).

Besides D1/D2 regions of LSU rRNA genes, phylogenetic analysis on ITS region was also made by Park and Jong (2003) to know the relationship among *Monascus* strains accessed from GeneBank. The phylogenetic tree constructed showed similar result with our analysis on ITS sequence of *Monascus* where *M. ruber* and *M. pilosus* were very closely related and all *M. purpureus* strains were included together in the same clade. Though it is still suggested that the two clades were very closely related, interestingly, all Indonesian *M. ruber* strains were in the same clade with *M. pilosus* AF451856. While *M. ruber* with accession number AF458470 retrieved from DDBJ was separated in another clade. This indicated that three *M. ruber* isolates and six white *Monascus* isolates showed little difference from *M. ruber* AF458470, but identical to *M. pilosus* AF451856 except for *Monascus* sp. KA30.1 and *M. ruber CKA3* with genetically little difference.

However, despite of this finding, it is still much surprising in regard with the outstanding morphological differences of several strains as mainly possessed by whitish strains such as *Monascus* sp. KA30.1, *Monascus* sp. MYOM, *Monascus* sp. KTB, *Monascus* sp. MYOT, *Monascus* sp. MM, *Monascus* sp. COEL. Park and Jong (2003) also reported similar result as their comparison of two very distinct species based on morphological observation such as *M. ruber* and *M. pilosus.* Therefore, we are in concordance with Park and Jo (2003) that it is needed to reveal in more details about molecular phytogeny to elucidate molecular taxonomy included in the identification *of Monascus* species.

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