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 sequenced. We also compared the sequence result with M. purpureus AF458473, M. ruber AF458470, M. kaoliang AF451859, M. araneosus 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 clade 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>
<thead>
<tr>
<th><em>Monascus</em> Strain</th>
<th>Isolation Year</th>
<th>Source of Isolation</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>M. purpureus</em> JMBa</td>
<td>2002</td>
<td>Fermented Chinese red rice</td>
</tr>
<tr>
<td><em>M. purpureus</em> JMS</td>
<td>2002</td>
<td>Powdered Medicinal Herb</td>
</tr>
<tr>
<td><em>M. purpureus</em> NGK-J</td>
<td>1995</td>
<td>Fermented Chinese red rice</td>
</tr>
<tr>
<td><em>M. purpureus</em> PKB1</td>
<td>2003</td>
<td>Fermented Chinese red rice</td>
</tr>
<tr>
<td><em>M. purpureus</em> PKB5</td>
<td>2003</td>
<td>Fermented Chinese red rice</td>
</tr>
<tr>
<td><em>M. purpureus</em> PRB1</td>
<td>2002</td>
<td>Fermented Chinese red rice</td>
</tr>
<tr>
<td><em>M. purpureus</em> SRBa</td>
<td>2002</td>
<td>Fermented Chinese red rice</td>
</tr>
<tr>
<td><em>M. ruber</em> CKA1</td>
<td>2003</td>
<td>Acid Liquor of <em>Arenga pinnata</em></td>
</tr>
<tr>
<td><em>M. ruber</em> CKA3</td>
<td>2003</td>
<td>Acid Liquor of <em>Arenga pinnata</em></td>
</tr>
<tr>
<td><em>M. ruber</em> SKF11</td>
<td>2003</td>
<td>Acid Liquor of <em>Arenga pinnata</em></td>
</tr>
<tr>
<td><em>Monascus</em> sp. COEL</td>
<td>1994</td>
<td>Invertebrate Specimen</td>
</tr>
<tr>
<td><em>Monascus</em> sp. KA30.1</td>
<td>1994</td>
<td>Invertebrate Specimen</td>
</tr>
<tr>
<td><em>Monascus</em> sp. KTB</td>
<td>1994</td>
<td>Invertebrate Specimen</td>
</tr>
<tr>
<td><em>Monascus</em> sp. MM</td>
<td>1994</td>
<td>Invertebrate Specimen</td>
</tr>
<tr>
<td><em>Monascus</em> sp. MYOM</td>
<td>1994</td>
<td>Invertebrate Specimen</td>
</tr>
<tr>
<td><em>Monascus</em> sp. MYOT</td>
<td>1994</td>
<td>Invertebrate Specimen</td>
</tr>
</tbody>
</table>
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'-TCCTCCGCTATTGATATGC-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 Univ19 (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 Univ19 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.

Molecular phylogeny

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 the six *Monascus* sp. strains which typically have whitish colonies, the three *M. rube* strains and *M. pilosus* AF451856. The similarities within the seven *M. purpureus* strains and *M. purpureus* AF458473, *M. araneosus* AF458471 and *M. kaoliang* AF451859 are very high more than 99% or almost 100%. However, *M. purpureus* 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 is shown by *Monascus* sp. KA30.1.

**DISCUSSIONS**

With regard to the level of very high similarity more than 99% almost near 100% among all *Monascus* fungi we analyzed, indicated that there is little mutation on ITS region of *Monascus* species. Concerning genetic diversity it seemed that there is a diverse mutation tendency as several *Monascus* strains such as
M. purpureus PKB1, M. purpureus PRBa, M. purpureus JMS, M. purpureus AF458473, M. ruber 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, Hawksworth 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, cleistothecium 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 phyteny to elucidate molecular taxonomy included in the identification of Monascus species.
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