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**MOLECULAR DELINEATION OF *Monascus* FROM CENTRAL LUZON,
PHILIPPINES BASED ON INTERNAL TRANSCRIBED SPACER (ITS) REGION**

**JAY LORENZ P. JOAQUIN¹, JIM ANDREUS V. MANGAHAS¹, JESUSA Q.
UNDAN², JERWIN R. UN DAN² AND RENATO G. REYES^{*1}**

1: Laboratory of Industrial Biotechnology and Bio-Industry,

**2: Laboratory of Molecular Biology and Biotechnology, Department of Biological
Sciences, College of Arts and Sciences, Central Luzon State University, Science City of
Munoz, Nueva Ecija, Philippines**

***Corresponding Author: Email: renato.reyes@clsu.edu.ph**

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ABSTRACT

Monascus purpureus is an edible fungus that is popularly used as natural colorant in the preparation of *buro*, a fermented rice and fish in the Philippines. The main purpose of this study is to distinguish and establish the phylogeny of the different strains of *Monascus* that were rescued in *Monascus* – coated rice (*angkak*), which were collected from the local markets in 7 towns (Aliaga, Guimba, Jaen, Natividad, San Ildefonso, Santo Domingo and Talavera) and 4 cities (Balanga, Cabanatuan, Gapan, San Jose) of Central Luzon region, Philippines. Cultures of *Monascus* were prepared following the standard protocol for fungal isolation. Following the manufacturer's protocol in DNA isolation and amplification, DNA of each sample was isolated and amplified with universal primers ITS3D and ITS4R and was confirmed using electrophoresis system. Basic Local Alignment Search Tool (BLAST) was used for species identification. Molecular Evolutionary Genetics Analysis version 6.06 software was used for phylogenetic analysis. Results of investigation revealed that *Monascus* in selected towns and cities in Central Luzon belonged to two species namely, *M. purpureus* and *M. kaoliang*.

Keywords: *angkak*, molecular identification, *Monascus*

INTRODUCTION

Monascus fermentation products (MFPs) have been widely used by people mainly in Asian countries for many centuries as food colorant, preservative, food supplement and traditional medicine [1]. With the culture of different strains of *Monascus* sp. and fermentation in various substrates, MFPs are widely available and are becoming popular worldwide [2]. In the Philippines, particularly in Central Luzon region, *Monascus* is being used as natural food colorant in the preparation of fermented rice and fish (locally known as *burong isda*). White rice grains which usually serve as carriers of *Monascus* is mixed with *burong isda* in order to improve the flavor and appearance. The reddish appearance of *burong isda* which is due to the pigment produced by *Monascus* makes it more appealing to local consumers in Central Luzon. Central Luzon is a landlocked region in Luzon Island, Philippines where the main agricultural activities are rice farming and inland fishing. Agricultural by-products such as rice and freshwater fishes serve as excellent ingredients in the preparation of *burong isda*. *Monascus* coated – rice which is locally known as *angkak* and

serves as starter for reddish pigment formation is commercially available in the local market in Central Luzon region. However, the species of the strains of *Monascus* that are being used remain to be unknown. Thus, there is a need to isolate and accurately identify the different strains in Central Luzon region. Isolation of strains is the particular interest because of the necessity to obtain *Monascus* with suitable kinetic characteristics for pigment cultivation [3]. Collection and identification of *Monascus* strains in Central Luzon region is essential in order to create data base which ultimately result in the establishment of *Monascus* germplasm collection. With the modern approaches based on DNA sequences, it is possible to accurately identify the unknown fungal species [4]. The assessment of microbial diversity in complex communities based on molecular methods have already been developed [5]. DNA analysis can reveal fungal diversity in ecosystems and offer the potential benefits of highly sensitive, rapid and accurate detection [6]. The species identification of fungi has been based mostly on the use of variable

ribosomal-DNA (rDNA) internally transcribed spacer (ITS) regions which generally provide greater taxonomic resolution than those from coding regions [7]. Moreover, DNA sequences in the ITS region are highly variable and might serve as markers for taxonomically - distant groups [8].

MATERIALS AND METHODS

Source of strain

Monascus – coated rice (*angkak*) collected from the local markets in 7 towns (Aliaga, Guimba, Jaen, Natividad, San Ildefonso, Santo Domingo and Talavera) and 4 cities (Balanga, Cabanatuan, Gapan, San Jose) of Central Luzon region, Philippines were processed in the Laboratory of Industrial Biotechnology and Bio-industry, Department of Biological Sciences, College of Arts and Sciences, Central Luzon State University, Science City of Munoz, Nueva Ecija, Philippines.

Rescue of mycelia

Mycelia were rescued and purified from *Monascus* - coated rice by inoculating aseptically the rice grain into the surface of the previously sterilized and plated potato dextrose agar, PDA (HiMedia Laboratories Pvt. Ltd, India). The

inoculated plates were incubated at ambient room temperature to allow the growth of the mycelia of *Monascus* (Figure 1).

Extraction of DNA

Fungal genomic DNA was extracted using GF-1 Nucleic Acid Extraction Kit (Vivantis Technologies, Malaysia). The fungal mycelia were grown on PDA slants for 10 days at 29°C - 32°C and were suspended in 400 µl of buffer C1. About 10 mg of fungal mycelia was scraped from fresh culture with a sterile nipper. The extracted DNA was kept at 4° C [2].

Amplification of DNA

PCR reactions were carried out in a 2720 thermal cycler (Applied Biosystem Inc, USA) with universal primers ITS3D and ITS4R. The amplification was performed in a 50 µl reaction mixture containing 33.96 ul of distilled water (RNase free), 1 µl forward primer (10 µl), 1 µl of reverse primer (10 µl) 5 µl of dNTP's (25 µl), 5mg of DNA template, 5 µl 10x buffer and .04 µl taq polymerase. The PCR products were confirmed by running the 1 µl of PCR product and the 1 kb DNA ladder stained with 1 µl gel red in 100 V for 30 mins using gel electrophoresis

system (Labnet International, Global). The PCR products were sent to 1st BASE laboratory, Malaysia for PCR purification and sequencing.

Sequencing

Direct DNA sequencing was performed using primers ITS3D. Sequences were assembled using Codon Code Aligner v 3.0 to estimate the quality of the generated sequences and to remove abnormal peaks of nucleotide for better identification. Basic Local Alignment

Search Tool (BLAST) using nucleotide sequence was used for sequence similarity and identification.

Phylogenetic analysis

Molecular Evolutionary Genetics Analysis version 6.06 software was used [9]. Three different phylogenetic trees were produced namely Neighbour Joining, Maximum Parsimony and Maximum Likelihood using default parameters of Molecular Evolutionary Genetics Analysis.

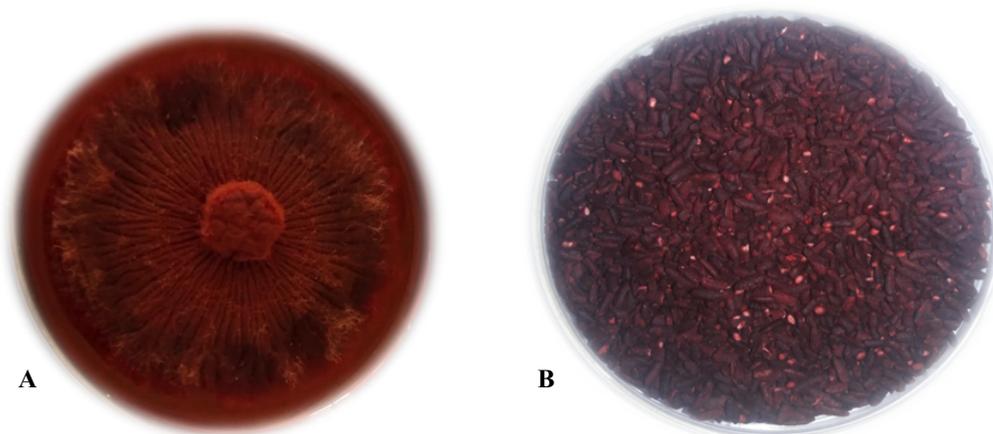


Figure 1: Cultural characteristics of *Monascus purpureus* in potato dextrose agar (A) and the angkak (B) as source of culture

RESULTS AND DISCUSSION

Table 1 shows that out of 11 samples, samples 1 and 2 were identified as *Monascus purpureus* with 99 % and 100% similarity to *M. purpureus* strain CICC40269 from the NCBI data base [10] respectively, while samples 3, 4, 5, and 6 were identified as *Monascus kaoliang*

with 99% similarity to *M. kaoliang* strains ATCC26264, ATCC36926, ATCC16367 and ATCC16361 [11] respectively. Moreover, samples 7, 9, and 10 were identified as *M. purpureus* with 100 % similarity to *M. purpureus* strain CMU004. On the other hand, samples 8 and 11 were identified as *M. purpureus*

with 100% similarity to *M. purpureus* strain CMU003[10]. This finding suggests that *Monascus* which are being sold in the local market came from different strains of *M. purpureus* and *M. kaoliang*.

Phylogenetic tree analysis

As shown in Figure 2, the phylogenetic trees show the relationship between 11 pre-identified taxa obtained from nucleotide sequences of DNA that were extracted from 11 different *Monascus* isolates using the primers ITS3-D and ITS4-R (with sequences ranging in size from 266 to 366 nucleotides) along with 13 *Monascus* strains (with sequences ranging in size from 272 to 617 nucleotides) that were gathered from Basic Local Alignment Search Tool (BLAST). The evolutionary distances were computed using the Maximum Composite Likelihood Method [9] and are in the units of the number of base

substitutions per site. The analysis involved 24 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 182 positions in the final dataset [9]. Surprisingly all *M. purpureus* strains shared the same node and clustered with all *M. kaoliang* strains and *M. auranticus* along with samples 1, 2, 3, 4, 5 and 6. While samples 7, 8, 9, 10 and 11 diverged from the group even having 99% similarity to *M. pupureus* CMU003 and CMU004 with accession number LC057319 and LC057320 (Figure 3).

Again, all *M. purpureus* strains shared the same node and clustered with all *M. kaoliang* strains and *M. auranticus* along with samples 1, 2, 3, 4, 5 and 6. Although samples 7, 8, 9, 10 and 11 diverged from the group even having 99% similarity to *M. pupureus* CMU003 and CMU004 with accession number LC057319 and LC057320.

Table 1: Species identity of *Monascus* collected in selected towns and cities of Central Luzon, region, Philippines

Sample	Source	Species	% Identity	NCBI Accession Code
1	Aliaga	<i>Monascus purpureus</i>	99	KP259283.1
2	Balanga City	<i>Monascus purpureus</i>	100	KP259283.1
3	Cabanatuan City	<i>Monascus kaoliang</i>	99	AB477252.1
4	Gapan City	<i>Monascus kaoliang</i>	99	AB477252.1
5	Guimba	<i>Monascus kaoliang</i>	99	AB477248.1
6	Jaen	<i>Monascus kaoliang</i>	99	AB477249.1
7	Natividad	<i>Monascus purpureus</i>	100	LC057320.1
8	San Ildefonso	<i>Monascus purpureus</i>	100	LC057319.1
9	San Jose City	<i>Monascus purpureus</i>	100	LC057320.1
10	Santo Domingo	<i>Monascus purpureus</i>	100	LC057320.1
11	Talavera	<i>Monascus purpureus</i>	100	LC057319.1

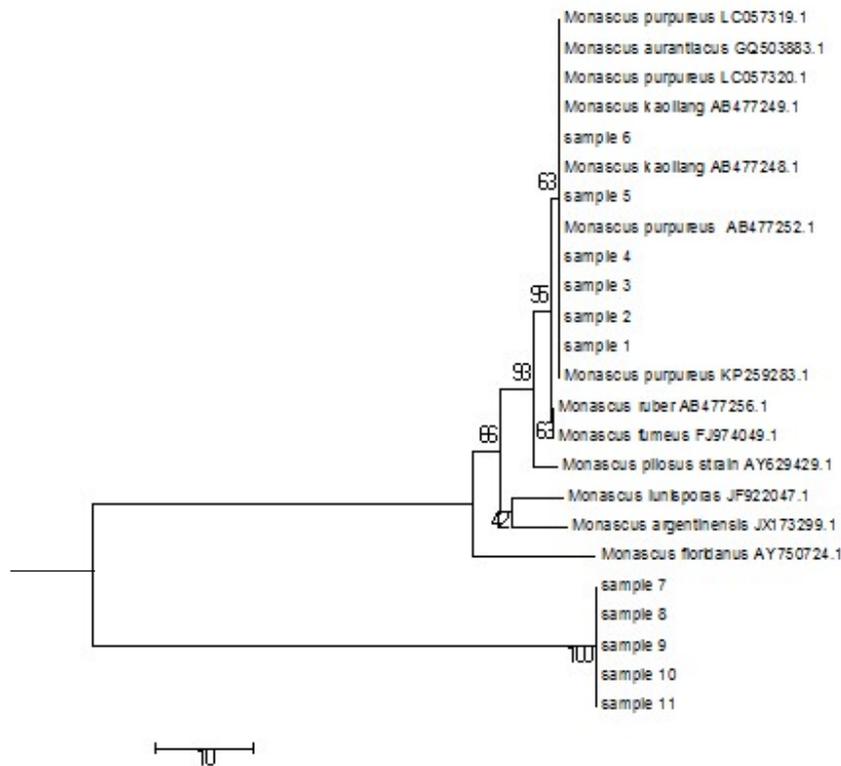


Figure 2: Molecular phylogenetic analysis by neighbour joining tree showing the relationship between 11 pre-identified taxa obtained from DNA of 11 different *Monascus* isolates using the primers ITS3-D and ITS4-R along with 13 *Monascus* strains gathered from Basic Local Alignment Search Tool (BLAST).

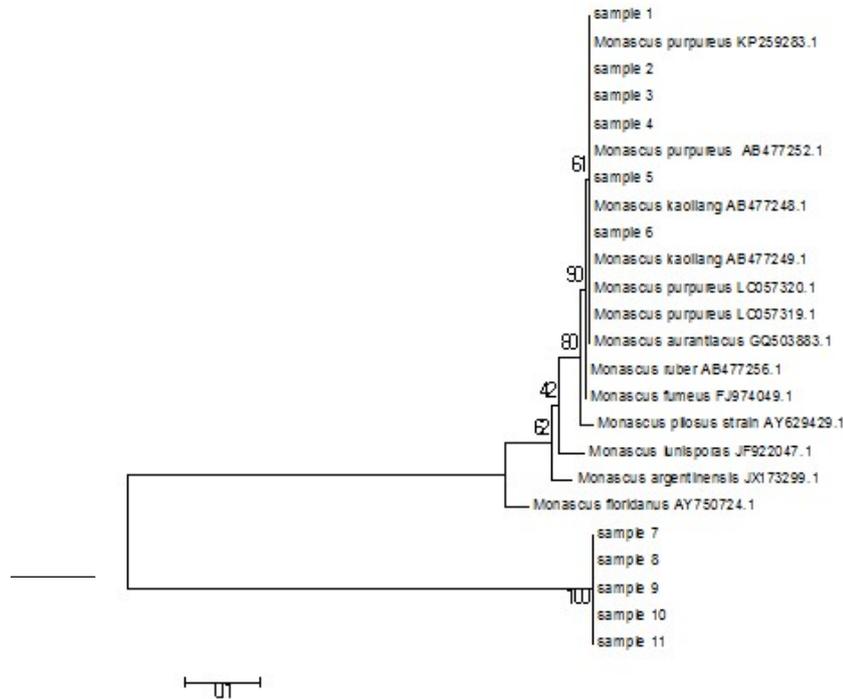


Figure 3: Molecular Phylogenetic analysis by Maximum Likelihood method showing the relationship between 11 pre-identified taxa obtained from DNA extracted from 11 different *Monascus* along with 13 *Monascus* strains gathered from Basic Local Alignment Search Tool (BLAST).

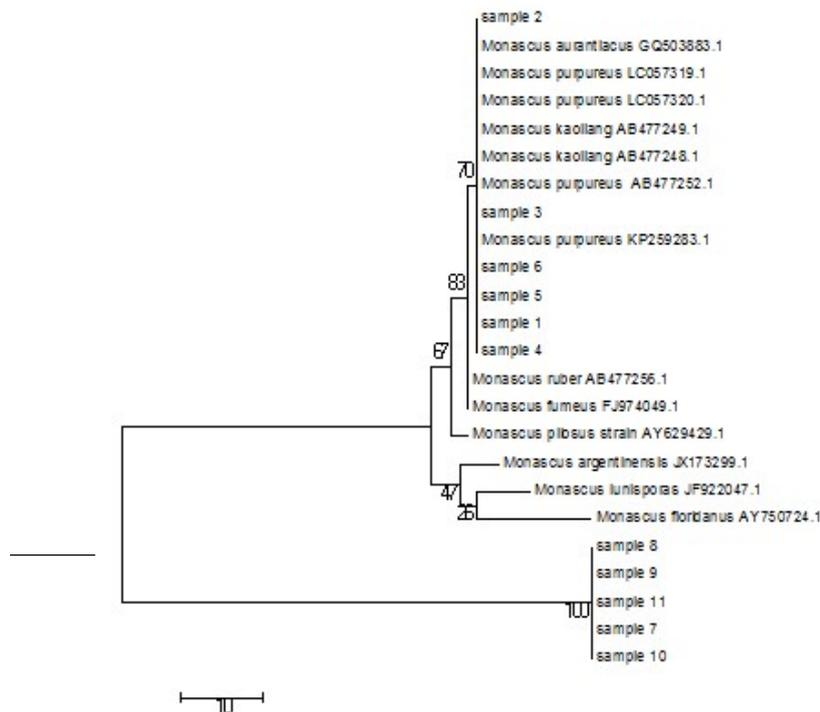


Figure 4: Molecular phylogenetic analysis by maximum parsimony analysis of taxa showing the relationship between 11 pre-identified taxa obtained from DNA extracted from 11 different *Monascus* isolates along with 13 *Monascus* strains gathered from Basic Local Alignment Search Tool (BLAST).

The evolutionary history was inferred using the Maximum Parsimony method (Figure 4). All positions containing gaps and missing data were eliminated. There were a total of 182 positions in the final dataset. Evolutionary analyses were conducted in MEGA6 [9]. Again, all *M. purpureus* strains shared the same node and clustered with all *M. kaoliang* strains and *M. aurantiacus* along with samples 1, 2, 3, 4, 5 and 6. Whereas samples 7, 8, 9, 10 and 11 diverged from the group even having 99% similarity to *M. purpureus* CMU003 and CMU004 with accession numbers LC057319 and LC057320.

In all phylogenetic trees, *Monascus floridanus* strain BCRC33310, *Monascus lunisporas* strain CBS 113675, *Monascus argentinensis* strain BCRC 33998, *Monascus pilosus* strain IFO 4487, *Monascus aurantiacus*, *Monascus ruber*, *Monascus fumeus* from GenBank were incorporated into the database. Individual BLAST searches with the sequences that caused gaps in the alignment in the ITS3 and ITS4 regions against the GenBank database, including the whole genome sequences, produced no meaningful similarity hits indicating that these

additional nucleotides are not associated with the currently known sequences. These results strongly suggest that insertions or deletions might have occurred independently without recombinations with already known, existing sequences. These insertions and deletions could lead to an inconsistent phylogenetic relationship with other targets. This finding strongly suggests that phylogenetic classification with ITS sequence information should be performed cautiously [12].

CONCLUSION

Based on the three different phylogenetic trees, this study showed a very high level of similarity of more than 99% which indicated that there is little mutation on ITS region of *Monascus* species. Regarding genetic diversity, it seemed that there is a diverse mutation tendency as several *Monascus* strains appeared differently though differences were slight. Therefore, this phylogenetic analysis revealed very close relationship among *Monascus* strains so we suggested that all *M. purpureus* were identical to each other. This work also suggested that *M. aurantiacus* and *M. kaoliang* ATCC are almost genetically the same species

with *M. purpureus* CICC because there are very little differences on their genetic makeup.

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