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PHENOTYPIC CHARACTERIZATION AND GENETIC VARIATION DETECTION USING ARBITRARY PRIMED POLYMERASE CHAIN REACTION FINGER PRINTING ON FOUR INDONESIAN WILD MONASCUS SPP

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Abstract

The aim of this study was to know the phenotypic characters and also genetic variation by using arbitrary primed polymerase chain reaction finger printing of four wild *Monascus* spp.

The phenotypic study showed *Monascus* sp. MYOT and *Monascus* sp. MYOM and *Monascus* sp. COEL had similar phenotypic characters. While *Monascus* sp. KTB was more different from the three *Monascus* isolates and considered different species. Although *M. pilosus* was regarded as the closest species but the four *Monascus* isolates were seemed to be new species.

The AP-PCR analysis of the four *Monascus* isolates produced 124 DNA bands recognized visually. The phylogenetic tree generated showed the differences among the four isolates studied. This study indicated that use of AP-PCR is more suitable for isolates or strains differentiation rather than species differentiation. Further molecular study is needed to be carried out to determine its species name for the four *Monascus* isolates such as by using ITS gene sequence or other structural genes.

Key words: *Monascus*, Wild, Phenotypic, Genetic, AP-PCR.

1. Introduction

Monascus is distributed worldwide (Young, 1930, Hawksworth and Pitt,1983). This fungus firstly was described in 1884 by van Tieghem (Young, 1930). *Monascus* species are commonly found in fermented foods, foodstuffs rich in starch, fruits with high water content, mouldy silage and soil (Domsch *et al*. 1980, Hawksworth and Pitt 1983). In common, the *Monascus* species show osmophilic affinity (Pitt and Hocking 1985). Several strains *Monascus* characterized by economical importance as its role in fermented food industry Western Asia mainly Chinese, Philippines, Japan, Thailand and Indonesia (Steinkraus *et al.*, 1983, Hesseltine 1965, Lin 1975), food colorants (Wong *et al*. 1981) and antibacterial activity (Wong andBau 1977).

M. purpureus was described firstly by Went in 1895. The origin of this fungus was from Chinese red rice in Java (Young, 1930).

This genus was revised in 1983 by Hawksworth and Pitt based on microscopic and cultural characters by observation on its growth on various media.

Biochemical studies were made by several scientists to identify *Monascus* species. Bridge and Hawksworth (1985) analyzed enzyme activity using APIZYM kit and Nishikawa *et al.* (1989) made analysis on composition of cellular fatty acid.

As other analysis biology molecular technique such as analysis protein profile, fatty acid content, isozyme, PCR, AFLP and RFLP (Lakrodi *et al.* (2000), RAPD can be used for knowing genetic relatedness rapidly and accurately, so this technique can be used for detection DNA polymorphism in many species from many organisms (plant, animal, man, bacteria and fungi). Implication of this technique is genetic marker since this technique can be used as molecular marker which makes significant impact in many biology fields (Gawel andBarlett, 1993).

Detected polymorphism by RAPD using short primers inherited in Mendelian pattern and can be made without information on DNA sequence. Polymorphism which is often useful as genetic marker can be easily detected using tracer labeled using fluorescence stain or by adding α-[33P] dCTP in PCR reaction. Polymorphism using RAPD is rapid and easy (Welsh *et al.,* 1991). Arbitrary primed polymerase chain reaction (AP-PCR) (Welsh andMcClelland 1990, Williams *et al*. 1990 was developed as an alternative which is simple and rapid method to obtain fingerprints of complex genomes.

Lakrodi *et al.* (2000) used RAPD method to detect the genetic variation within 25 isolates *Monascus* isolated from Chinese red rice and sofu. They suggested that there was a relative narrow genetic source on the *Monascus* used for food product in Asia.

This study will aim at knowing phenotypic character among four *Monascus* isolates and also their genetic variation using AP-PCR. These isolates were originated from animal wet specimens in Indonesia.

2. Materials and Methods

2.1. *Monascus* Isolates Studied

Monascus strains were used were four isolates which were isolated from wet animal specimens from Bogor, Indonesia. The four isolates were *Monascus* sp. MYOM, *Monascus* sp. MYOT, *Monascus* sp. KTB and *Monascus* sp. MM.

2.2. Phenotypic Characterization

Phenotypic characterization based on microscopic and cultural observation on growth on malt extract agar 2% which incubated at 25° C for seven days.

2.3. Cultivation and Purification of *Monascus* for DNA extraction purpose

All fungi for were cultivated on Yeast Malt (YM) Agar plate. While for fungal 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 and 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.

2.4. DNA extraction

Before freezing by pouring liquid nitrogen and grounded by mortar and pestle, water excess was removed from the harvested mycelial mass of each fungus by putting on to paper.

To do DNA extraction, QIAamp tissue kit (QIAGEN) was used. Quantification and Qualification by both spectrophotometer and gel electrophoresis were carried out.

2.5. Primer

Primer used in this study was primer AP1 5'ATGCAGGAGTCGCAT-3'.

2.6. AP-PCR amplication

The procedure of AP-PCR amplication was essentially accordingly to Welsh and McClelland (1990). Firstly, 400 μL of reaction mixture [10 mM Tris (pH8.3), 50 mM KCl, 0.01 % gelatin, 5 mM $MgCl_2$ 0.2 mM each of dATP, dCTP, dGTP, and dTTP, 10 μM primer, 10 U of AmpliTaq DNA polymerase (Perkin Elmer Cetus)] was prepared. This mixture was divided into aliquots, frozen rapidly in liquid nitrogen and stored at -80°C was subjected to PCR. PCR was conducted in 0.5 mL tubes using the program Temp Control System PC-700 (ASTEC). In each tube, 4 μL of reaction mixture and 1 μL of DNA sample were mixed and overlaid by mineral oil. The temperature profiles was four cycles of 94°C for 1 min, 40 °C for 5 min and 72°C for 5 min followed by 30 cycles of 94°C for 1 min, 60 °C for 1 min and 70°C for 1 min. At the end of this reaction, 20 μL of second PCR mixture [10 mM Tris (pH8.3), 50 mM KCl, 0.01 % gelatin, 1.5 mM $MgCl_2$ 0.2 mM each of dATP, dCTP, dGTP, and dTTP, 92.5kBq 0.5 of [α-^{32}P] dCTP, 0.5 U of AmpliTaq DNA polymerase] was added to tube mixed well by pipetting and subjected to an additional 20 cycles of 94°C, 60 °C and 72°C as above.

2.7. Electrophoresis and autoradiography

The amplicons were denatured with formamide and electrophoresed in standard 5% acrylamide 0.5 TBE 45% urea sequencing gels (40 cm long) at a constant power of 80 W for 100 min, cooled with a water cooling plate and a fan. The gels were then scanned using Hitachi Image Analyzer FM Bio II.

3. Result and Discussion

Table 1. Phenotypic characters observed of four *Monascus* spp. Isolates Based on Growth on MEA 2% incubated at 25° C for seven days.

Monascus Isolates	Colony Colour	Colony Diameter (mm)	Aerial Mycelia	Ascomata	Ascospores		Soluble Pigment
					Size (μm)	Shape	
COEL	White	31-32	Slight (+)	Hyaline	4-7 X 4	Ellipsoid	Red
MYOT	White	31-33	Little (++)	Hyaline	4-7 X 4	Ellipsoid	Red
KTB	White	40-47	Little (++)	Hyaline	6 X 3	Ellipsoid	Red
MYOM	White	30-37	Slight (++)	Hyaline	4-7 X 4	Ellipsoid	Red

Table 2. Scoring data from bands recognized visually. - = not observed, + = observed.

Isolate	Band no.																													
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
MYOM	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	-	-	-	+	-	-	-	+	-	-	-	-	-	-	-
KTB	-	+	+	-	+	-	+	+	-	+	+	-	+	+	+	+	-	-	-	+	+	+	+	+	+	+	+	-	+	-
MYOT	+	-	-	+	-	+	-	-	+	-	-	+	-	-	-	-	+	+	-	-	+	-	+	-	+	-	-	-	-	+
COEL	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	+	-	-	-	+	-	-	-	-	-	-	+	-	-

Isolate	Band no.																													
	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60
MYOM	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
KTB	-	+	-	-	-	-	+	-	-	-	-	-	-	+	-	+	+	+	+	+	+	-	-	-	-	+	+	-	-	-
MYOT	+	-	+	+	-	-	-	+	+	+	-	+	+	+	-	+	+	-	-	-	-	-	+	+	+	-	-	+	-	+
COEL	+	-	-	-	+	+	-	-	+	+	+	-	+	+	+	-	+	+	-	+	+	+	+	-	-	-	-	-	+	+

Isolate	Band no.																													
	61	62	63	64	65	66	67	68	69	70	71	72	73	74	75	76	77	78	79	80	81	82	83	84	85	86	87	88	89	90
MYOM	-	-	-	-	-	-	-	-	-	+	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
KTB	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	+	+	-	-	-	+	+	+	+	-	+	-	+	-	+
MYOT	+	+	-	+	-	+	+	+	-	-	-	-	+	-	+	-	-	+	-	+	-	+	-	-	-	-	-	-	-	-
COEL	-	-	+	+	+	-	-	-	+	-	+	+	-	-	-	+	+	+	+	-	-	-	-	-	+	+	+	-	+	-

Isolate	Band no.																													
	76	77	78	79	80	81	82	83	84	85	86	87	88	89	90	91	92	93	94	95	96	97	98	99	100					
MYOM	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-					
KTB	+	+	-	-	-	+	+	+	+	-	+	-	+	-	+	-	+	+	-	-	-	-	-	-	-					
MYOT	-	-	+	-	+	-	+	-	-	-	-	-	-	-	-	-	-	-	+	-	-	+	+	-	+					
COEL	+	+	+	+	-	-	-	-	-	+	+	+	-	+	-	+	-	-	-	+	+	-	-	+	+					

Isolate	Band no.																							
	101	102	103	104	105	106	107	108	109	110	111	112	113	114	115	116	117	118	119	120	121	122	123	124
MYOM	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	+
KTB	+	+	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	+	+
MYOT	+	+	-	+	+	-	-	+	+	-	-	-	-	-	-	+	+	+	-	-	-	-	-	-
COEL	-	-	+	-	-	-	-	-	-	+	+	+	+	+	+	-	-	-	+	+	-	-	-	-

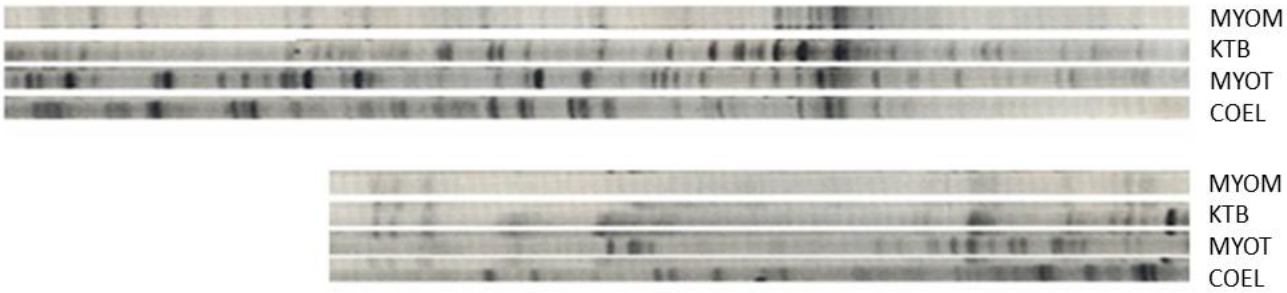

Figure1. Photography of DNA Bands on Electrophoresis Gel Produced by AP-PCR of Four *Monascus* Isolates.

Table 3. Distance Matrix of The Four *Monascus* Isolates Generated Based on Neighbor-Joining/UPGMA Analysis Method.

***Monascus* Isolates**	**Distances**			
	MYOM	KTB	MYOT	COEL
MYOM	0.00000			
KTB	0.50187	0.00000		
MYOT	0.59476	0.99852	0.00000	
COEL	0.62235	0.93558	0.85786	0.00000

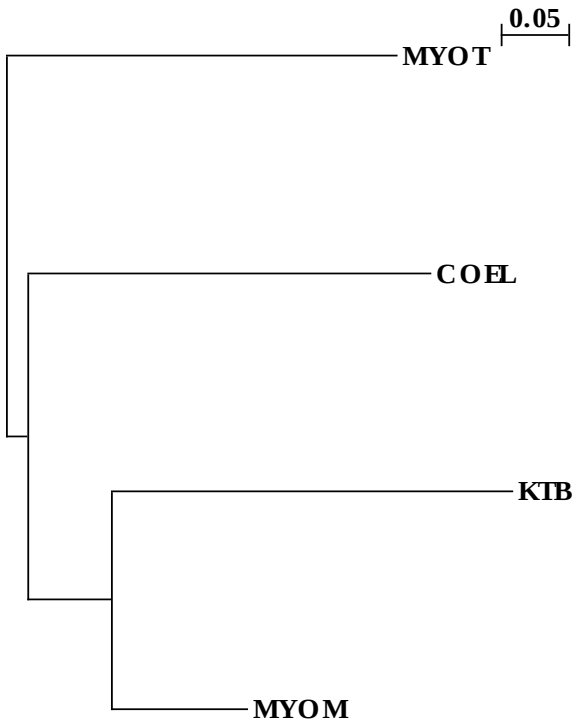

Figure 2. Phylogenetic tree of the four *Monascus* Isolates which generated using UPGMA Method from Phylip 3.68 v.

The result showed that based on phenotypic characterization, the three isolates were very similar. While KTB isolate was different from the three The three isolates had the same size of ascospore 4-7 x 4 μm and KTB isolate had different size 6 x 3 μm (Table 1.).

After following the identification key to *Monascus* species by Stchigel *et al.* (2004), the determination of four *Monascus* isolated did not meet with any species included in the key because there were rather different of its ascospore size and soluble pigment. However, it was indicated that the closest species was *M. pilosus* based on many phenotypic characters was resemblance with this species. The fusiform *aleurioconidia* was found from *Monascus* sp. MYOM and *Monascus* sp. MYOT was questionable for taxonomic determination as *M. pilosus* had no this *Basipetospora* form. *M. pilosus* had obpyriform to globose of its *Basipetospora* form. So, it was suggested to take it into consideration in

taxonomic determination of those two isolates. Park and Jo (2003) also reported similar result as their comparison of two very distinct species based on morphological observation such as *M. ruber* and *M. pilosus.*

The electrophoresis produced many amplified DNA fragments as shown as DNA bands. After analysis, a number of 124 DNA bands were detected and determined visually included several smears based on photograph taken (Figure 1).

After scoring based on presence or absence band (Table 2), phylogenetic analysis was made using Phylip 3.68 v by using DNAdist and Neighbour UPGMA. Data obtained was then analyzed using DNAdist in PHYLIP (*Phylogeny Inference Package*) versi 3.68 v (Joseph Felsenstein, the University of Washington, USA). DNAdist was used to analyze matrix distance. UPGMA method from neighbor then was used to gain phylogenetic tree as shown on Figure 2.

Distance matrix generated by using neighbor-joining/UPGMA analysis method showed the four *Monascus* spp. Isolates were different (Table 2) and the phylogenetic tree showed separation the four *Monascus* spp. (Figure 2). Table 3 showed genetic distance among the four *Monascus* spp.. So, it is genetically the four *Monascus* spp. were different. However, this result could not be used in species differentiation as the nature of AP-PCR methods is producing randomized amplication as use of randomized oligo primer. In this study, AP-PCR method applied showed many DNA bands that can be used for comparison among isolates or strains. The AP-PCR product also can be used as a finger printing for *Monascus* isolates/strains collected.

Similar genus with *Monascus* is *Xeromyces* L.R. Fraser which differs based on its sesil ascomata, two spores ascus, plano-convex ascospore and xerophilic. This genus has a taxomomical change with including *X. bisporus* L.R. Fraser ke into *Monascus* genus based its high homology on its ITS sequence with the closest of member *M. purpureus*, *M. lunisporas* (Stchigel *et al.* 2004). Based on his result on phylogeny study and together with morphological data, Stchigel *et al.* (2004) have the same perception with Park and Jong (2003) and agree with the combination proposed by von Arx in 1970. Based on analysis on phylogenic tree generated, Stchigel *et al.* (2004) suggested that four known *Monascus* species are synonym, such as *M. pilosus, M. purpureus, M. ruber, M. sanguineus* and synonymized species of *M. purpureus* (*M. anka*, *M. araneosus* and *M. kaoliang*) are the same species as it is supported by 99 % bootstrap based on sequence analysis of ITS region. He also suggested that more studies were needed to could confirm the possible synonymy of these taxa as *M. ruber* Tiegh. based on other structural genes (18 rDNA, β-tubulin, etc.). Park and Jo (2003) suggested that to reveal this *Monascus* taxonomy further molecular study is needed to get more detail about molecular phylogeny so as to elucidate molecular taxonomy of *Monascus* species.

This study revealed that further molecular taxonomic study of the four *Monascus* spp. to clarify its species statue. Moreover variation among isolates showed by their AP-PCR finger printing which indicated genetic differences among the *Monascus* four isolates studied.

4. Conclusion

The four *Monascus* spp. were seemed to be new species although they were similar with *M. pilosus* which regarded as their closest species.

More over more variation among the four isolates was indicated by their AP-PCR finger printing which showed genetic differences among the *Monascus* four isolates studied. This study revealed that further molecular taxonomic study of the four *Monascus* spp. to determine its species name. While AP-PCR analysis showed its useful for differentiation among the four *Monascus* spp..

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