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Genetic diversity of the pathogenic fungus *Sarocladium oryzae* causing sheath rot on rice using rep-PCR

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Abstract. Rice becomes the main commodity cultivated in Asia. Sheath rot disease becomes one of the challenges in increasing rice production. *Sarocladium oryzae* is known to be one of the major pathogens in causing sheath rot disease. This pathogen has been reported in several rice production countries, such as Brazil, India, Taiwan, Philippines, Thailand, including Indonesia, and causing yield loss of up to 85%. The study of genetic diversity is important to investigate the rapid shift in genetic structures. A total of six *S. oryzae* strains showed diverse in molecular using repetitive-PCR (rep-PCR). The isolate SO8 and SO13 isolate SO2 and SO11 and isolate SO3, and SO5 has the relatively same the band patterns in BOX, ERIC, and REP sets of primers. The results of the UPGMA analysis show that *S. oryzae* in the various primers can be divided into three different group consistency. This result indicates that the rep-PCR method can genetically distinguish *S. oryzae*. The use of the three main sets, BOX, ERIC, and REP, suggested differentiating the other fungal species.

1. Introduction

Rice is the primary agricultural product cultivated in Asian countries. Rice demand continues to rise year after year, as the population increases. However, due to biotic and abiotic factors, there are several obstacles in the effort to increase the productivity of rice. One of the biotic factors that play a role in inhibiting rice productivity is pathogenic infections that cause sheath rot. After being first isolated in Taiwan in 1922 [1], [2], *Sarocladium oryzae* was considered the first major fungal pathogen that caused rice sheath rot disease. *S. oryzae* has been reported in several rice production countries, such as Brazil, India, Taiwan, Philippines and Thailand [5] including in Indonesia [3], [4]. In some countries, sheath rot disease has caused a yield loss of up to 85% [5]. The disease affects the quantity and quality of rice [6].

The polymerase chain reaction (PCR) was used to diagnose and characterize fungal species for molecular recognition of pathogens [7]. Molecular technique based DNA are also gradually becoming the tool of choice for quantifying and interpreting genetic diversity and phylogeny of fungal species [8]. There are scattered, repeated sequences in the prokaryotic and eukaryotic genomes that distinguish



longer single DNA sequences [9]. BOX [10], ERIC [11], [12], and REP [12], [13] are examples of repeated evolutionary DNA sequences. Repeated elements of PCR fingerprint polymorphisms (rep-PCR) have become a tool frequently used to identify bacterial species that evaluate the distribution in the prokaryotic genome of repeated DNA sequences [12]. rep-PCR for repetitive elements in the eukaryotic genome has also been developed [14]. rep-PCR is a simple method for distinguishing closely related microbial strains, inferring phylogenetic relationships, and investigating their diversity in different ecosystems [15]. These studies aimed to (i) to determine the genetic diversity of *Sarocladium oryzae* using rep-PCR, and (ii) to evaluate the effectiveness of rep-PCR using repetitive primers BOX, ERIC, and REP in determining the molecular diversity of *Sarocladium oryzae*. The research findings will be the basis for developing rep-PCR techniques in determining the diversity of a fungus.

2. Material and methods

2.1 Fungal strains

This study used *Sarocladium oryzae* strains from the previous study by Pramunadipta *et al.*, [3] (Table 1).

Table 1. *Sarocladium oryzae* strain used on this study.

Species	Strain	Origin	Location	GenBank accession no.	Reference
<i>Sarocladium oryzae</i>	SO2	<i>Oryza sativa</i>	Serang, Banten, Indonesia	MT012231	[3]
	SO3	<i>Oryza sativa</i>	Sumedang, West Java, Indonesia	MT012232	[3]
	SO5	<i>Oryza sativa</i>	Pati, Central Java, Indonesia	MT012234	[3]
	SO8	<i>Oryza sativa</i>	Gowa, South Sulawesi, Indonesia	MT012236	[3]
	SO11	<i>Oryza sativa</i>	Karawang, West Java, Indonesia	MT012233	[3]
	SO13	<i>Oryza sativa</i>	Tulungagung, East Java, Indonesia	MT012235	[3]

2.2 Genomic DNA extraction

The isolates obtained were then conducted genomic DNA extraction. *Sarocladium oryzae* were then cultured into PDA for 5 days. Three plugs (diameter \pm 5 mm) of *S. oryzae* isolates were added into 50 ml PDB (potato dextrose broth) and grown at 25 °C for 3-4 days. Mycelia of fungal species were harvested for molecular identification. 0.5 mg of each dried fungal mycelia was extracted using a CTAB procedure [16] with modification. In short, fungal mycelia cell walls were broken down in the presence of sterile quartz sand by grinding with glass rods. The CTAB 2% (CTAB, EDTA 100 mM, NaCl 1,4 M, Tris HCl 50 mM, β -mercapto-ethanol 1%, sterile water) extraction buffer was then added, and purification with chloroform:isoamyl alcohol (24:1) and 96% alcohol precipitation was performed after incubation at 65 °C. The DNA then resuspended in 50 μ l of pure water.

2.3 PCR amplification and visualization

Each PCR mixed contained 12.5 μ l of PCR mix (dNTPs; MgCl₂; 2x MyTaq™ HS Red Mix, Bioline), 1 μ l of each primer (100 μ M), 1 μ l of genomic DNA (ng/ μ l) and ultrapure water in a total volume of 25 μ l. The rep-PCR using BOX, ERIC, and REP primers with the PCR conditions conducted in Table 2. Reactions were performed in BioRad T100™ Thermal Cycler (Bio-Rad, Hercules, CA, USA).

The PCR products were then visualized using 1% agarose gel (0.4 g of agarose (Molecular Biology Grade, Vivantis) in 40 ml of TBE 1X) and added 4 µl agarose gel staining (ViSafe Red Gel Stain, Vivantis). Agarose gel was then electrophoresed at 100 V for 35 min using BioRad Mini ReadySub-Cell GT Horizontal Electrophoresis Cell (Bio-Rad, Hercules, CA, USA). Agarose gel was then visualized under UV light. The DNA bands were then captured using a digital camera.

2.4 Data analysis

The band patterns were analyzed by summarizing them into Table 0-1 (0 for no band, 1 for the present band). Table 0-1 was made using Microsoft Excel to place the band amplification in columns and isolate numbers in rows to construct the dendrogram. Dendrogram that represents the genetic relationship between *S. oryzae* was generated from the similarity matrices by the use of unweighted pair-group arithmetic (UPGMA) mean methods in numerical taxonomy and multivariate analysis systems (NTSYSpc version 2.10e) [19]. The JPEG file format is then export using Adobe Illustrator 2020 software.

Table 2. Primer sets for rep-PCR used in this study.

Primers	Sequence	PCR cycle		No. of cycles	Reference
		Temp	Time		
BOX A1R	5' CTACGGCAAGGCGACGCTGACG 3'	95 °C	7 min	30	[17, 18]
		94 °C	1 min		
		53 °C	1 min		
		65 °C	8 min		
		65 °C	16 min		
ERIC 1	5' ATGTAAGCTCCTGGGGATTACAC 3'	95 °C	7 min	30	[17, 18]
		94 °C	1 min		
		52 °C	1 min		
		65 °C	8 min		
		65 °C	16 min		
ERIC 2	5' AAGTAAGTGACTGGGGTGAGCG 3'	94 °C	7 min	35	[17, 18]
		94 °C	1 min		
		40 °C	1 min		
		65 °C	8 min		
		65 °C	16 min		
REP1R	5' IIIICGICGICATCIGGC 3'	94 °C	7 min	35	[17, 18]
REP2-1	5' ICGITTATCIGGCCTAC 3'	94 °C	1 min		
		40 °C	1 min		
		65 °C	8 min		
		65 °C	16 min		

3. Results and discussion

3.1 rep-PCR primers polymorphism patterns

In this study, the genetic diversity fingerprints of *S. oryzae* strain from rice sheath rot disease were investigated through three sets of primer, BOX, ERIC, and REP fingerprints gave successful amplification. The polymorphic bands ranging between 100 bp – 4000 bp were observed. The DNA fingerprints differed from one another. On the basis of the banding patterns of PCR products, the differences between strains have been visually evaluated. A total of 59 bands ranging from 200 bp - 3000 bp were generated by BOX-PCR. For SO11, the maximum number of bands (11) was observed, while the lowest (9) was observed for SO3 and SO5 (Figure 1A). ERIC-PCR identified a total of 72 bands with the band sizes ranging from 100 bp – 4000 bp. SO3 showed the highest number of the band (15), while SO8 and SO 13 showed the lowest band (9). Therefore, the primer set ERIC was unable to generate thick and clear bands pattern for strain SO8 and SO13 (Figure 1B). For rep-PCR, 64 bands were performed with the band size ranging from 100 bp – 4000 bp. The highest number of bands (13) for SO8 was observed, while the lowest bands (8) were observed on SO13 (Figure 1C). The rep-PCR fingerprints showed a high genotypical diversity for all *S. oryzae* strains in all of these PCRs.

The genomic fingerprinting based on PCR is a reliable and effective technique for microbial species discrimination [20]. rep-PCR is a valuable molecular tool for identifying and differentiating bacterial and fungal organisms [21]. This result shows that rep-PCR was able to produce DNA banding patterns in *S. oryzae* strains. DNA band patterns showed relatively different patterns between strains tested and each primer used. These results indicate that rep-PCR can differentiate the strain of *S. oryzae* tested molecularly. Several studies have been conducted to apply rep-PCR to differentiate plant-pathogenic fungi. Olive and Bean [22] refer to fingerprinting with rep-PCR as an efficient technique for identifying and analyzing *Tilletia* species by phylogenetic relationships. In other studies, the effectiveness of rep-PCR fingerprinting and more practical insight into rep-PCR validity is clearly demonstrated by Abdollahzadeh and Zolfaghar [23] as a rapid and accurate method for detecting and differentiating the species of Botryosphaeriaceae. Aguilar-Hawod *et al.*, [24] used rep-PCR to differentiate *Fusarium oxysporum* f. sp. *cubense* (Foc) Tropical Race 4 (TR4).

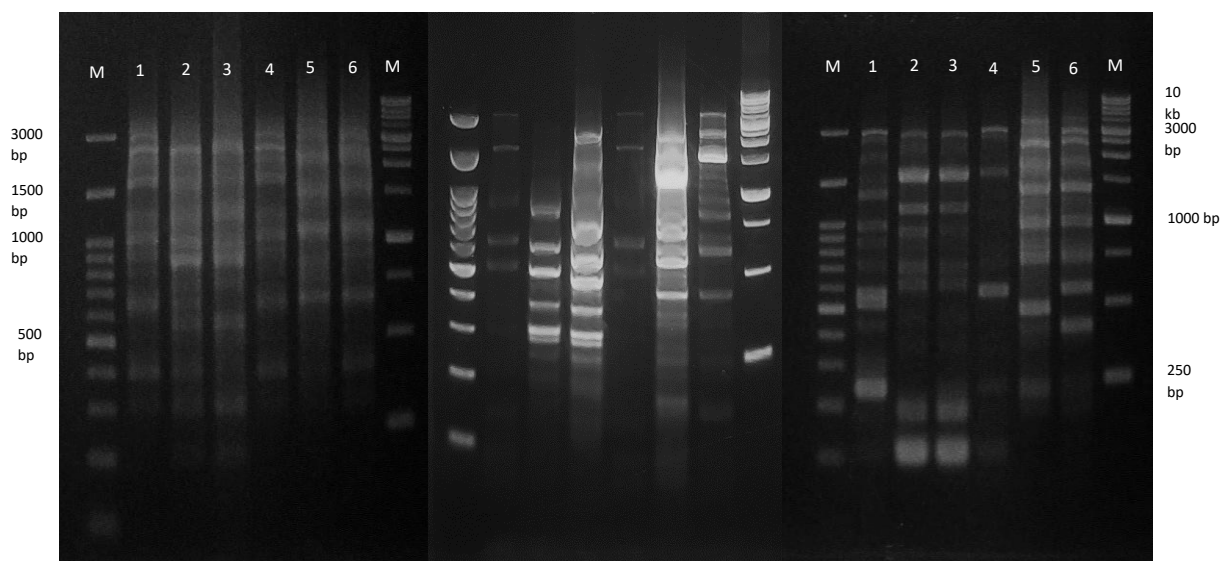


Figure 1. DNA fingerprints generated with primer sets BOX (A); ERIC (B); and REP (C) for *Sarocladium oryzae* strains. lane 1: SO8, lane 2: SO11, lane 3: SO2, lane 4: SO13, lane 5: SO3, lane 6: SO5, M. left 100 bp ladder Geneaid, M. right 1kb ladder Geneaid.

3.2 Genetic similarity analysis

The result of rep-PCR amplification showed that isolate SO8 and SO13 isolate SO2 and SO11 and isolate SO3, and SO5 has the relatively same pattern in all sets of primers (Figure 1). To determine the interaction of the isolates, the dendrogram was reconstructed on the basis of the BOX, ERIC, and REP PCR fingerprint bands. Using Jaccard similarity coefficients and UPGMA clustering methods, the data was analyzed. The dendrogram generated by the fingerprints of BOX, ERIC, and REP PCR revealed three major clusters containing two strains at 65% similarity (Figure 2). The clustering was apparent and represented three distinct *S. oryzae* clusters. The result of data analysis of three primers showed that isolate SO8 and SO13, isolate SO2 and SO11, and isolate SO3 and SO5 has consistency result in the same cluster in the dendrograms (Figure 2).

Analysis of the clusters is a valuable presentation method for evaluating the relationships between and within species. The UPGMA dendrograms generated were comparable and useful for the study of diversity for *S. oryzae* using the rep-PCR method. This research shows that rep-PCR can group strains of *S. oryzae* into three groups genetically based on the resulting banding pattern. This result indicates that the strain of *S. oryzae* tested varied molecularly. A thorough understanding of pathogen

populations and species-level diversity is important because high genetic diversity suggests a rapid shift in genetic structures. This mechanism shows the development of more virulent organisms and strains in response to changing ecosystem management practices and improved the biological fitness of those organisms [25]. Masanto *et al.*, [18] applied to the rep-PCR and SSR markers as a helpful tool for researching the genetic diversity of *Phytophthora palmivora*. Genetic diversity studies on *S. oryzae* have been carried out using ISSR markers [26], and SNP markers [27, 28]. The results of the current study show the effectiveness of fingerprinting with rep-PCR and a more realistic insight into the validity of rep-PCR as a method for the rapid and precise differentiation of strains of *S. oryzae*. The development of rep-PCR as a method for determining genetic diversity between species needs to be done using this method to differentiate another plant pathogenic fungal species in order to further validate the rep-PCR. Genetic differences research results can be used for the basis of developing *S. oryzae* control methods in the field.

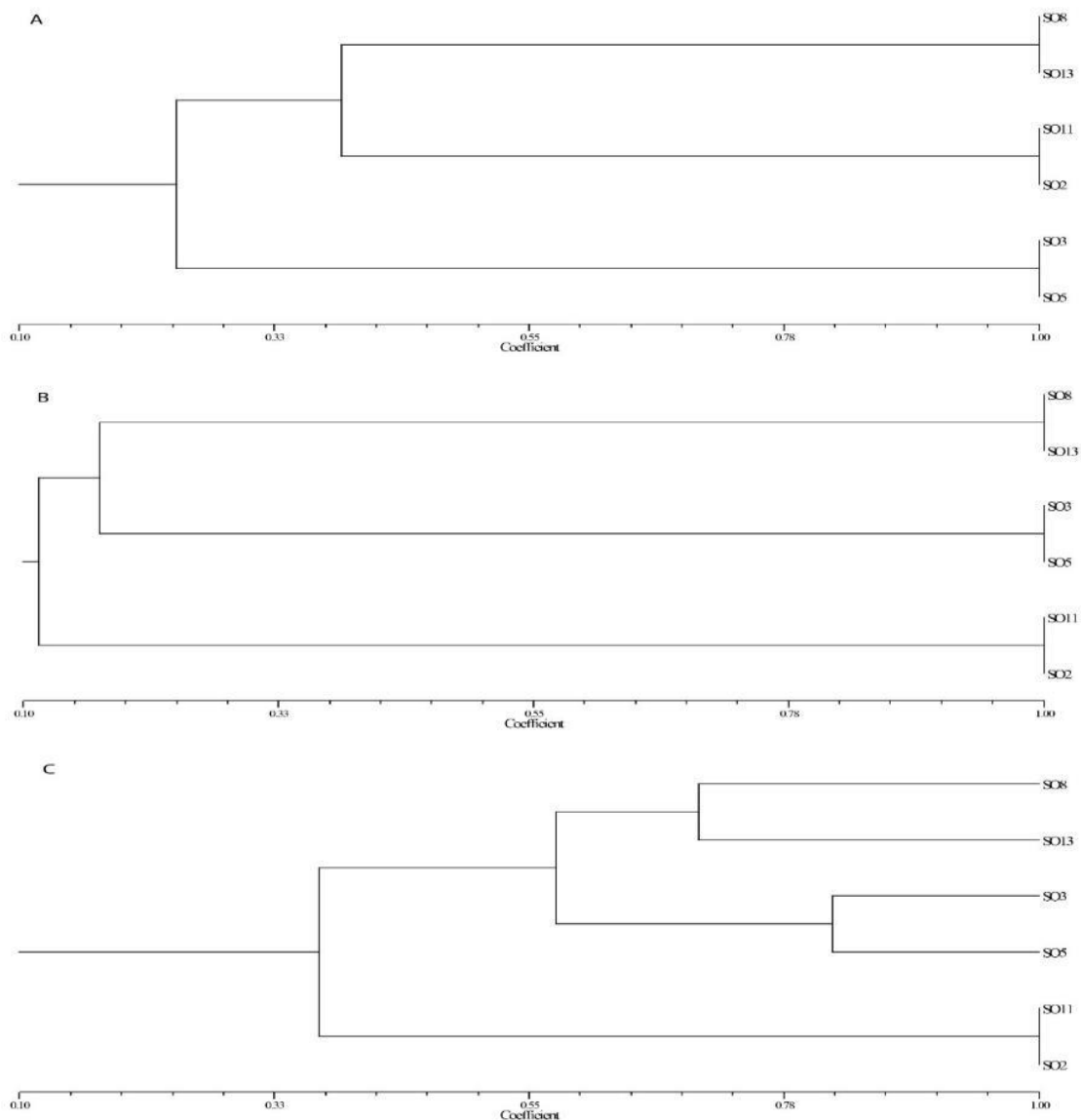


Figure 2. Dendrogram resulting from UPGMA cluster analysis of DNA banding profiles generated with primer BOX (A); ERIC (B); and REP (C) for *Sarocladium oryzae* strains.

4. Conclusions

The use of rep-PCR can genetically distinguish *Sarocladium oryzae* isolates. The band patterns and results of the UPGMA analysis show that *S. oryzae* in the various primers can be divided into three different group consistently. All three primer sets of BOX, ERIC, and REP are suggested to be used for accurate typing and differentiation of the other fungal species.

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