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Morphological and molecular identification of *Pyricularia oryzae* causing blast disease on rice (*Oryza sativa*)

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ABSTRACT

Aims: Rice (*Oryza sativa*) is one of Malaysia's most significant crops. Rice blast caused by *Pyricularia oryzae* is one of the most serious diseases of *Oryza sativa*, causing significant damage to the Malaysian rice crop and impacting productivity. This study was carried out to isolate and characterize phytopathogenic fungal isolates associated with rice blast collected in a paddy field in Alor Setar, Kedah, Malaysia.
Methodology and results: Morphological characterization of seven fungal isolates obtained showed thin, white, and grayish green mycelia and the reverse colony was light yellow to brown. The fungal isolates produced two-septate pyriform (pear-shaped) conidia with solitary, unbranched and light brown conidiophores. Pathogenicity tests of all isolates on rice leaves revealed diamond-shaped symptoms with a grayish center and brown edge. All isolates showed similar morphological and pathogenicity characteristics; thus, a representative isolate was further identified through DNA sequencing and phylogenetic analysis of the internal transcribed spacer (ITS) region for species confirmation. Based on DNA sequences of ITS and phylogenetic analysis, the representative isolate was confirmed as *P. oryzae*.
Conclusion, significance and impact of study: Seven isolates morphologically identified as *Pyricularia* sp. were tested as pathogenic by causing rice blast disease. Representative isolate P2 (USM-PD1) was confirmed to be *P. oryzae* by DNA sequencing and phylogenetic analysis of the ITS region. This study provides information on the etiology and symptomatology of rice blast disease caused by *P. oryzae* USM-PD1 that can be applied to diagnose and mitigate the threat posed by this plant pathogen for the disease management.

Keywords: Morphological and molecular identification, *Oryza sativa*, phylogenetic analysis, *Pyricularia oryzae*

INTRODUCTION

Rice is a critical staple food for more than half of the world and its production must double by 2050 to keep up with food demand from population increase (Nalley *et al*., 2016). The Malaysian food security policy is primarily concerned with guaranteeing the availability, accessibility and utilization of rice to the community. Since the 1970s, three food security policy objectives have been established: to provide a high price for paddy farmers producing rice, to achieve a set level of rice self-sufficiency and to ensure a consistent and high quality of rice to consumers (Bala *et al*., 2014). Malaysia's proactive and progressive measures to promote paddy and rice sector development have continued throughout the Eleventh Malaysian Plan (2016-2020) and the National Agro-Food Policy (2021-2030). Rice diseases are among the challenges in rice cultivation that lead to significant economic losses and a decrease in the national self-sufficiency level (SSL) (Firdaus *et al*., 2020). Rice blast disease caused by the ascomycete fungus, *Pyricularia oryzae* Carava [teleomorph: *Magnaporthe grisea* (Herbert) Barr] is one of the key restrictions causing significant harm to rice production in Malaysia (Longya *et al*., 2020; NurulNahar *et al*., 2020). According to Muda Agricultural Development Authority (MADA), Malaysia, rice blast caused by *P. oryzae* is one of the main diseases that infect paddy crops and affect yield productivity in Malaysia. Moreover, rice blast is the most destructive disease in almost all rice-growing countries (Shahriar *et al*., 2020). Rice blasts are reported to cause considerable harvest losses, particularly in Asia, namely China, Sri Lanka, Indonesia, Bangladesh and India. In addition, the disease is also a major problem in South America, Australia, Korea and the Philippines (Gupta *et al*., 2021).

The blast fungus, *P. oryzae* attacks rice plants at all stages of growth, causing spindle-shaped lesions on the leaves and panicle neck rot at the culm (Rijal and Devkota, 2020). In Malaysia, there are two forms of blast

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diseases: foliar blast, which infects the seedling stage and panicle blast, which infects panicles during the reproductive stage (NurulNahar *et al*., 2020). Infection at the foliar level frequently impacts rice plant tillering, resulting in fewer panicles per hill. Meanwhile, symptoms of panicle blast occur at the panicle's base, resulting in rotten neck or neck rot. Seeds, crop residues and secondary hosts are possible sources of *P. oryzae* inoculum. A complete cycle of blast diseases begins when the spores infect and cause symptoms such as lesions or spots on the rice plants and then the fungus begins sporulation and releases new spores into the air. The pathogen survives as airborne conidia, where one cycle can take a week to complete and one lesion can produce more spores after twenty days (Sopialena and Palupi, 2017).

Rice blast outbreaks are a persistent problem in all rice-growing regions across the world (Nalley *et al*., 2016). One of the management measures to tackle the outbreak for each season is the breeding of blast resistant varieties (Pooja and Katoch, 2014; Rijal and Devkota, 2020). Since 1965, the development of rice varieties has been developed by the Department of Agriculture (DOA), Malaysia. Then, the breeding was taken over by MARDI and different varieties of rice were reported including Mahsuri, Malinja, Setanjung, MR 219, MR 220, MR 253, MR 263 and MARDI Siraj 297. MADA and MARDI have recently reported MR 315 as a new variety of rice (NurulNahar *et al*., 2020).

Sampling of rice plants showed the occurrence of rice blast symptoms in the rice field in Alor Setar, Kedah. Diseased samples were transported to the laboratory for further investigation. The focus of the study is to characterize fungal isolates associated with rice blast disease through morphological, molecular and pathogenicity analyses.

MATERIALS AND METHODS

Sample collection and isolation of fungi

Sampling of rice plants showing symptoms of rice blast disease was carried out in Alor Setar, Kedah. Sampling was conducted in a specific rice field in Alor Setar, Kedah, Malaysia (6.15742° N, 100.38066° E) as the aim was to isolate specific target pathogens causing severe rice blast in that region, according to MADA. Further study will be carried out towards treating the fungus with a biological control agent. The samples were transported to the Bioprocess Laboratory, Universiti Sains Malaysia and further screened for the isolation of fungi causing the disease. A total of eight *O. sativa* plants were sampled consisting of 17 blast leaves. Figure 1 shows the diamond-shaped symptomatic leaves with a grayish center and brown edge observed in the field. Small pieces of leaves (1 cm^2) from the edge of symptoms were cut, each piece bearing healthy and diseased parts and subjected to surface-sterilization method. The pieces of leaves were submerged in a sterilized beaker containing 70% alcohol and 1% sodium hypochlorite for 3 min each, then were rinsed three times in sterile distilled water for 1 min each and dried on sterilized filter paper. The sterilized leaves were subsequently placed on potato dextrose agar (PDA) plates and incubated at room temperature, 25 ± 2 °C for 3 to 5 days until visible mycelia appeared. The grown mycelia were subcultured on new PDA and incubated at 25 ± 2 °C for 7 days. The single spore technique was conducted to obtain pure cultures of fungal isolates.

Figure 1: Rice blast symptoms on leaves of *O. sativa* observed in the fields.

Morphological characterization of fungal isolates

The fungal isolates were cultured on PDA and incubated at 25 ± 2 °C. Macroscopic characteristics such as colony appearance, pigmentation and mycelial growth rate of isolated fungi were observed and measured. A 7-day-old fungal pure culture from PDA was aseptically punched with a cork borer (0.6 cm) to form a mycelial disc. The mycelial disc was transferred to a new PDA plate and incubated for 7 to 14 days at 25 ± 2 °C. The appearance of colonies from the upper and lower surfaces was observed. Moreover, the mycelial growth rate of each isolate was measured in cm/day. Microscopic characteristics including size and shape of conidia and conidiophore formation were observed using a light compound microscope (Olympus, Model BX41).

Pathogenicity test

The pathogenicity evaluation was conducted in the plant house of the School of Biological Sciences, Universiti Sains Malaysia for 7 days. All fungal isolates were grown on PDA plates and incubated for 7 days at 25 ± 2 °C. Mycelial plugs of all fungi (0.6 cm) served as an inoculum, while PDA plugs served as controls. Three replicates for each fungal isolate were performed on the same leaf. All target plant parts were sterilized with 70% alcohol and wounded with sterile toothpicks. The mycelial and control plugs were inoculated onto wounded parts and wrapped with wet cotton wool to retain moisture. A cellophane tape was placed on the tested part to hold the cotton and mycelial plug. Lesion formation was observed daily until day 7. Lesion area (cm^2) and disease severity index (%)

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Table 1: *Pyricularia* species and outgroup used in phylogenetic analysis.

Species	Isolate	Host	Locality	GenBank accession no. ITS	References
Pyricularia grisea	MaG	*Eleusine coracana*	Peradeniya, Sri Lanka	MW136319	Dissanayake *et al.* (2022)
	C2P21B.2	*Glycine max*	Minas Gerais, Brazil	JQ936264	Leite *et al.* (2012)
P. oryzae	CBS 433.70	*Oryza sativa*	Denmark	MH859782	Vu *et al.* (2019)
	CBS 365.52	*O. sativa*	Japan	MH857082	Vu *et al.* (2019)
	UPM-PO	*O. sativa*	Selangor, Malaysia	KT693184	Awla *et al.* (2017)
	PO-FA01	*O. sativa*	Selangor, Malaysia	KM249937	Abed *et al.* (2014)
	IC1	*O. sativa*	Corrientes, Argentina	OQ552821	Bastida *et al.* (2023)
	IC2	*O. sativa*	Corrientes, Argentina	OQ552822	Bastida *et al.* (2023)
	USM-PD1	*O. sativa*	Kedah, Malaysia	OR133216	Present study
P. penniseticola	BF0017	*Pennisetum typhoides*	Burkina Faso, Kamboinse	KM484925	Klaubauf *et al.* (2014)
	ML0031*	*P. typhoides*	Longorola Sikasso, Mali	KM484929	Klaubauf *et al.* (2014)
	CD0143	*Digitaria exilis*	Odienne, Cote d'Ivoire	KM484927	Klaubauf *et al.* (2014)
P. pennisetigena	ML 0036*	*Pennisetum* sp.	Cinzana, Mali	KM484935	Klaubauf *et al.* (2014)
	BR0067	*Cenchrus echinatus*	Imperatriz, Brazil	KM484931	Klaubauf *et al.* (2014)
	BR0093	*Echinochloa colona*	Primeiro de Maio, Brazil	KM484932	Klaubauf *et al.* (2014)
Pyricularia sp.	P180	*Setaria viridis*	South Korea	MK430967	Choi (2019)
	MC1	*Stenotaphrum secundatum*	New Zealand	AY265323	Bussaban *et al.* (2005)
P. urashimae	CPC 29421	*Chloris distichophylla*	Londrina, Parana, Brazil	KY173439	Crous *et al.* (2016)
	CPC 29419	*Panicum maximum*	Aral Moreira, Mato Grosso do Sul, Brazil	KY173438	Crous *et al.* (2016)
	CPC 29414*	*Urochloa brizantha*	Amambai, Mato Grosso do Sul, Brazil	KY173437	Crous *et al.* (2016)
Gaeumannomyces radicicola	CBS 149.85	*Zea mays*	South Africa	KM484844	Klaubauf *et al.* (2014)

*Ex-type strains.

were calculated based on Li *et al*. (2011). The scoring of blast disease severity was evaluated based on the protocol provided by the International Rice Research Institute (IRRI). IBM SPSS® Statistics 27 was used to perform statistical analysis of the data obtained. To detect significant differences in lesion area, one-way ANOVA was performed, and means were compared with Tukey's test. Differences were considered significant when $p<0.05$.

Molecular characterization and phylogenetic analysis

A representative isolate P2 (USM-PD1), morphologically identified as *Pyricularia* sp. was further confirmed by molecular identification. The fungal isolate was grown in 10 mL of potato dextrose broth (PDB) in universal bottles and incubated at 25 ± 2 °C for 7 days. The fungal mycelia grown at the surface of the PDB were harvested and dried on sterile filter paper. The fungal mycelia were then homogenized with liquid nitrogen and squeezed using a sterile mortar and pestle until a fine powder formed. The fine powder of the mycelia was transferred into a 1.5 mL centrifuge tube. Then, the genomic DNA of the fungal isolate was extracted using the Invisorb Spin Plant Mini Kit (Stratec Biomedical AG).

The primer pair of ITS1 (TCCGTAGGTGAACCTGCGG)/ITS4 (TCCTCCGCTTATTGATATGC) was used in this study (White *et al*., 1990). PCR was carried out in a total volume of 50 µL containing 8 µL Green GoTaq buffer (5×; Promega), 8 µL $MgCl_2$ (25 mM; Promega), 1 µL dNTP (10 mM; Promega), 8 µL each primer (2.5 µL for each ITS; Integrated DNA Technologies), 0.3 µL Taq DNA polymerase (5 U/µL; Promega), 0.6 µL genomic DNA and 16.1 µL of sterile distilled water. For control, 49 µL of PCR mix without added DNA was prepared. PCR was performed using a PCR machine (MyCycler thermal cycler, Bio-Rad) under the following conditions: initial denaturation at 95 °C for 3 min, followed by 35 cycles of denaturation at 95 °C for 30 s, annealing at 52 °C for 45

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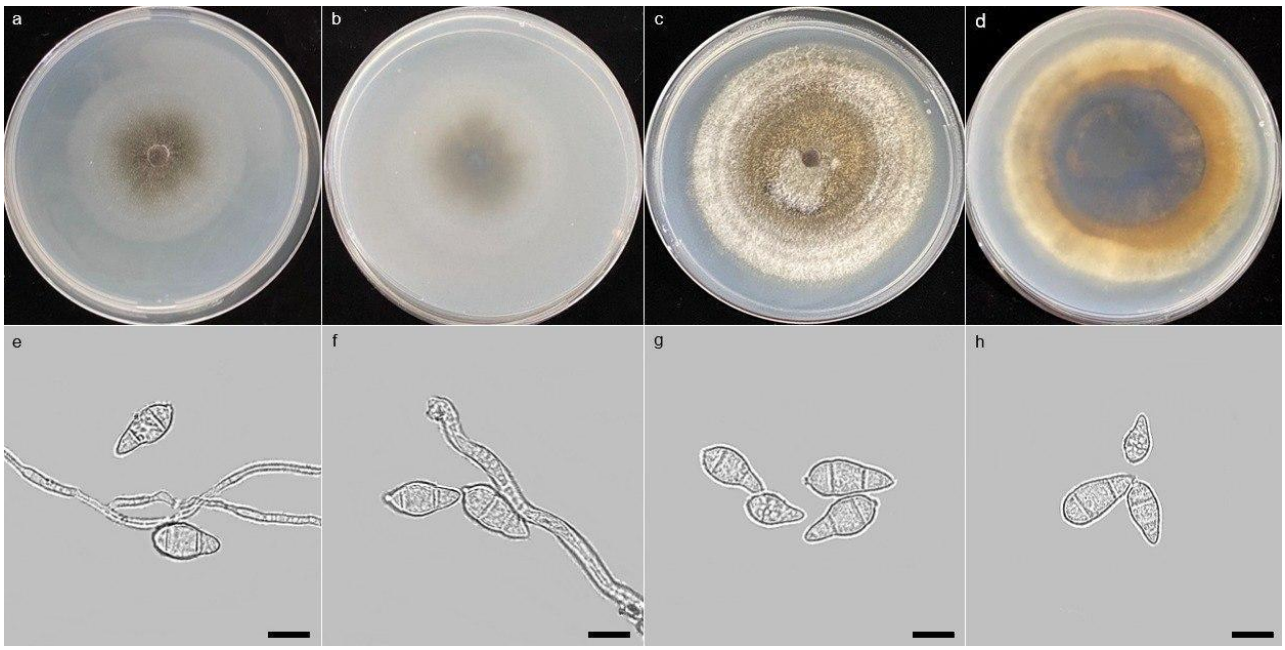

Figure 2: Morphological characteristics of *P. oryzae* (isolate P2, USM-PD1). (a, c) Upper surface of the colony on PDA after 7 and 14 days; (b, d) Lower surface of the colony on PDA after 7 and 14 days; (e, f) Mycelia, conidiophores and conidia; (g, h) Conidia. Scale bars of e to h = 10 µm.

sec, extension at 72 °C for 1 min and 30 sec and lastly final extension at 72 °C for 10 min. The PCR products were then run in 1% agarose gel electrophoresis at 110 V and 240 mA for 45 min. A Thermo Scientific DNA ladder with 100 bp was used as a marker to estimate the size of the amplified PCR products. The PCR products were sent to the service (First BASE Laboratories Sdn Bhd) for DNA purification and sequencing.

The obtained DNA sequences were aligned in MEGA 11 (Tamura *et al*., 2021). Consensus sequence obtained was compared with other sequences in the GenBank database by Basic Local Alignment Search Tool (BLAST) and deposited into GenBank for accession number. A phylogenetic tree of the maximum-likelihood (ML) was conducted using MEGA 11. Kimura 2-parameter was selected as the best model in constructing the phylogenetic tree of *P. oryzae* in the present study with 1000 bootstrap replications (Kimura, 1980). The isolate of the present study and the references used in constructing the phylogenetic tree are shown in Table 1.

RESULTS

Isolation and morphological characterization of fungal isolates

A total of seven isolates of *Pyricularia* sp. were obtained from rice leaves with blast symptoms in Alor Setar, Kedah. Based on morphological characteristics, all isolates showed similar colony morphology with thin white and grayish green mycelia on the upper surface of the colony (Figure 2a and 2c), whereas light yellow to brown on the lower surface of the colony (Figure 2b and 2d). The growth rate was 0.65 ± 0.01 cm/day. The conidia produced by the isolates were two-septate pyriform (pear-shaped), narrowed at the tip and rounded at the base, and 23.86 ± 0.32 µm × 7.64 ± 0.16 µm (Figure 2e to 2h). The conidiophores produced were solitary, unbranched, light brown, and 1.46 ± 0.18 µm (Figure 2e and 2f). All morphological characteristics were compared with previous studies as described in the discussion.

Pathogenicity test

The pathogenicity tests performed on healthy rice plants (*O. sativa*) revealed that all seven isolates were tested as pathogenic. Control leaves remained healthy after 7 days (Figure 3a). However, fungal-inoculated leaves exhibited diamond-shaped symptoms with a grayish center and brown margin after 3 days of incubation similar to those observed in the field (Figure 1 and 3b). As progressed, the lesions expanded longitudinally surrounded by a yellow halo (Figure 3c and 3d). Koch's postulates were fulfilled as the same fungus was re-isolated from the inoculated leaves with blast symptoms.

The lesion area produced on inoculated rice leaves was between 5.7 ± 0.3 to 8.0 ± 0.5 cm^2 (Table 2). The disease severity index ranged from 62.96 ± 3.2 to 88.88 ± 5.6%, of which the score was greater than 51% in the IRRI blast disease severity scale. There were significant differences between isolates in terms of lesion area and disease severity index. Despite significant differences among isolates, all isolates demonstrated pathogenicity on rice leaves and the same symptom when compared to

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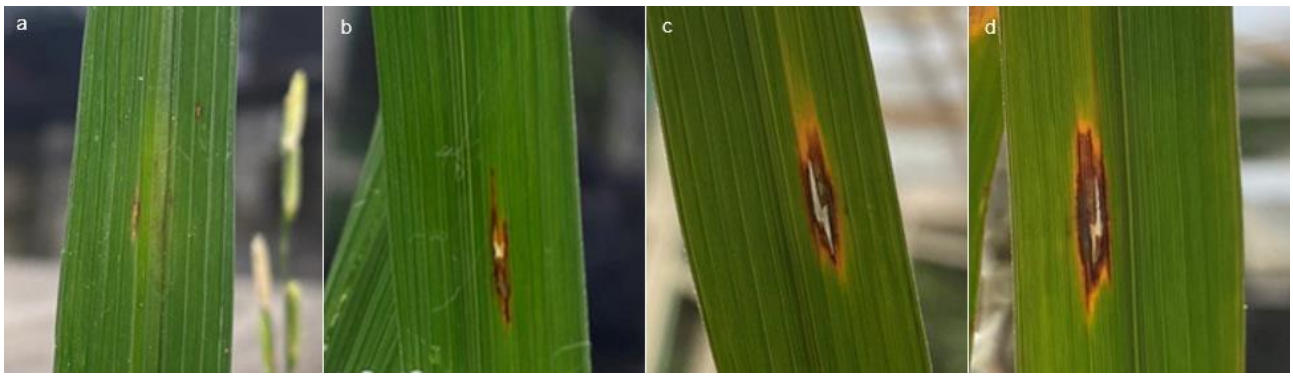

Figure 3: Pathogenicity test of *P. oryzae* (isolate P2, USM-PD1) on leaves of *O. sativa*. a) Control; b) 3 days; c) 5 days; d) 7 days after incubation.

Table 2: Lesion area and disease severity index (%) of *O. sativa* caused by *P. oryzae* isolates after 7 days of inoculation.

Isolate code	*Lesion area (cm^2)	*Disease severity index (%)
PI	7.8 ± 0.3^{bc}	87.04 ± 3.2^{bc}
P2 (USM-PD1)	8.0 ± 0.5^{b}	88.88 ± 5.6^{b}
P3	6.8 ± 0.3^{cd}	75.93 ± 3.2^{cd}
P4	6.8 ± 0.6^{cd}	75.93 ± 6.4^{cd}
P5	6.2 ± 0.6^{de}	68.52 ± 6.4^{de}
P6	5.7 ± 0.3^{e}	62.96 ± 3.2^{e}
P7	6.7 ± 0.3^{de}	74.07 ± 3.2^{de}
Control	0^{a}	0^{a}

*Mean ± SD, followed by different letters within the column is significantly different ($p \leq 0.05$) according to Tukey's test.

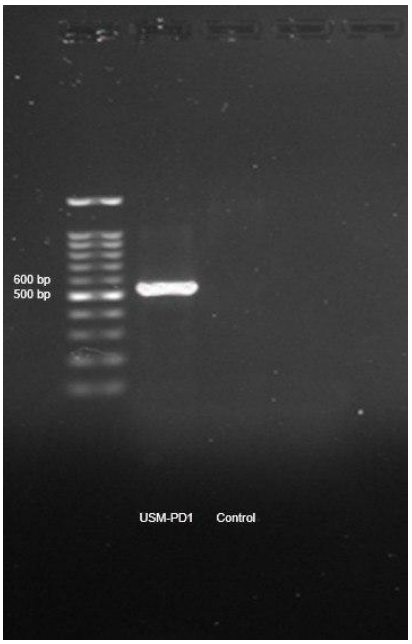

Figure 4: Gel electrophoresis of amplified ITS region of a representative isolate P2 (USM-PD1). First line lane indicates 1 kb DNA ladder and last lane indicates control (without DNA).

isolated symptoms in the field. Isolate P2 (USM-PD1) showed a larger lesion area and the highest disease severity index compared to others, thus, it was selected as a representative for further molecular identification and phylogenetic analysis.

Molecular characterization and phylogenetic analysis

A representative isolate, P2 (USM-PD1) was confirmed as *P. oryzae* based on molecular identification by the internal transcribed spacer (ITS) region. PCR amplification of the ITS region generated a single band of approximately 540-550 kb (Figure 4). According to the BLAST search, isolate P2 (USM-PD1) showed 100% identity similar to the reference sequence MH859782 (Vu *et al*., 2019) and KT693184 (Awla *et al*., 2017).

The ML analysis was performed to delineate the phylogenetic relationship of isolate P2 (USM-PD1) along with 19 reference isolates of *Pyricularia* and one outgroup (Figure 5). Isolate P2 (USM-PD1) clustered in the same group with the other five reference isolates of *P. oryzae* isolated from *O. sativa* from Malaysia and other countries with a bootstrap value of 90%.

DISCUSSION

Rice blast is the most devastating disease in all rice growing seasons in the world. *P. oryzae* is a filamentous fungus responsible for causing blast in rice fields. The

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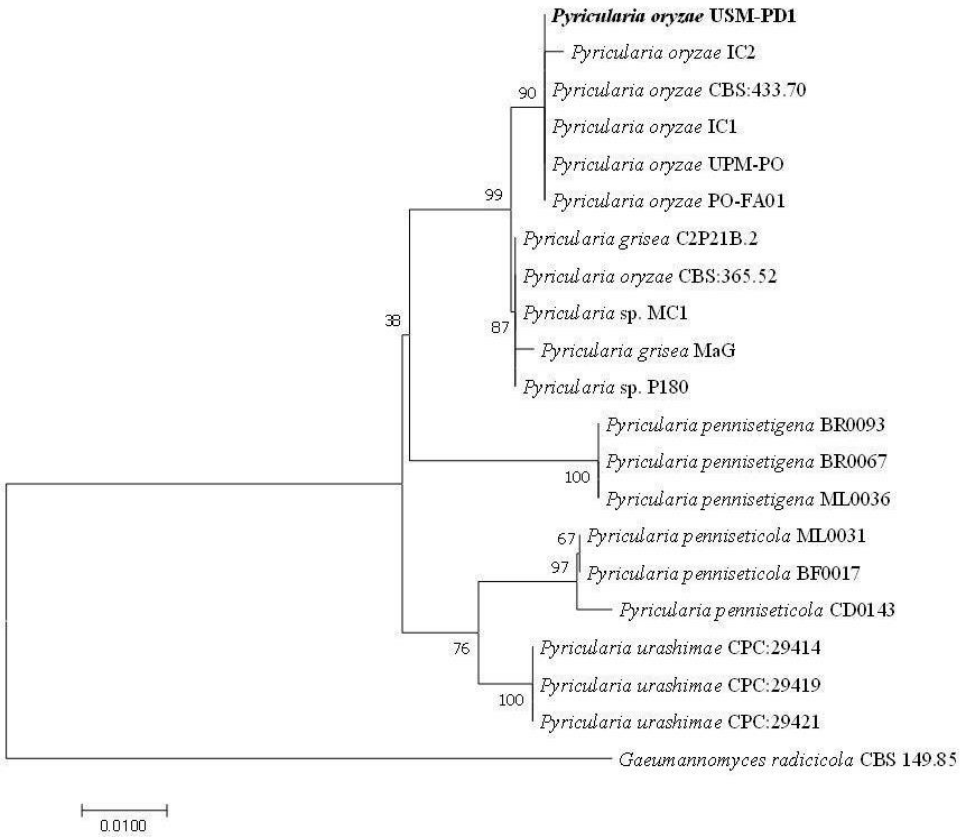

Figure 5: Phylogenetic relationship obtained from the maximum-likelihood (ML) analysis of the internal transcribed spacer (ITS) based on the Kimura 2-parameter model. The sequence obtained from the present study is bolded and *Gaeumannomyces radicicola* represents the outgroup. Scale bar indicates the number of nucleotide substitutions per site.

present study recovered seven isolates of *P. oryzae* from Alor Setar, Kedah and their pathogenicity was confirmed through pathogenicity tests, causing blast disease on rice (*O. sativa*). The symptoms developed as diamond-shaped with a grayish center and brown margin, recognized as a typical rice blast symptom. The lesion morphology was also reported as a water-soaking gray lesion, as a primary lesion then continued to form an older lesion as a white gray diamond-shaped and encircled with red brown (Srivastava *et al*., 2014; Shahriar *et al*., 2020).

The morphology of all isolates was characterized based on upper and lower colonies, growth rate, conidial size and conidiophores. The colony appearance described in the present study was in accordance with studies by Al Noman *et al*. (2021) and Hussin *et al*. (2020). Moreover, the colony growth of all *P. oryzae* isolates grown on PDA plates was 4.87 ± 0.02 cm on day 7, which was in line with the findings by Al Noman *et al*. (2021) and Castroagudín *et al*. (2016) that stated *P. oryzae* can grow in a range of 4.3 cm and reach up to 8.4 cm on day 14.

The size and shape of conidia are the key parameters for the classification and identification of Pyriculariaceae. The conidial shape of Pyriculariaceae varies greatly depending on the host (Longya *et al*., 2020). The conidial features in this study showed similarities with Al Noman *et al*. (2021), Castroagudín *et al*. (2016) and Klaubauf *et al*. (2014). These studies stated pyriform shaped conidia reported as *P. oryzae* with the conidial size in the range of 17-28 µm × 6-8 µm. Moreover, conidiophores were solitary, unbranched, and light brown comparable to Hosseini-Moghaddam and Soltani (2013), stating that conidiophores were slightly brown, rarely branched and suggested sympodial growth.

Pathogenicity tests revealed the development of rice blast symptoms by all isolates of *P. oryzae*. The growth rate of each isolate determines its virulence. The disease severity index (%) was calculated for each isolate using the IRRI procedure (Chandler, 1982). Based on the pathogenicity tests conducted, *P. oryzae* isolates, able to penetrate the host through artificially created wounds. According to Qi *et al*. (2019) and Tan *et al*. (2023), *P. oryzae* can infect the damaged hosts via appressoria. The disease cycle begins when a conidium attaches to the host surface by conidial tip mucilage formation. This is followed by a succession of developmental processes, including germination, germ tube growth, appressorium creation, emergence of a penetration peg from the

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appressorium and finally invasive growth in the host (Money, 2016).

Morphological and pathogenicity tests indicated that all seven isolates were identified as *Pyricularia* sp. Morphology alone is frequently insufficient to resolve the identity of *Pyricularia* until species due to overlapping morphological characteristics among species (Srivastava *et al*., 2014). Therefore, ITS DNA sequencing was employed to identify the species of one representative isolate. ITS is a universal fungal barcode and is widely used as a molecular tool for the identification and phylogenetic analysis of diverse fungal species (Raja *et al*., 2017). The ITS region has been used for the detection, identification, and classification of *Pyricularia* isolates (Longya *et al*., 2020). In this study, universal primers ITS1 and ITS4 were used to amplify the ITS region. To support the findings, the consensus sequence of isolate P2 (USM-PD1) of *P. oryzae* was further compared in the phylogenetic analysis (Figure 5). In addition to being used in identification, phylogenetics also plays a role in inferring evolutionary relatedness among groups of organisms through their similarities and differences (Das *et al*., 2014). The identity of the isolate P2 (USM-PD1) was resolved and identified as *P. oryzae* through DNA sequences and phylogenetic analysis.

CONCLUSION

Seven fungal isolates were morphologically identified as *Pyricularia* sp. responsible for causing rice blast disease. Representative isolate P2 (USM-PD1) was confirmed to be *P. oryzae* by DNA sequencing and phylogenetic analysis of the ITS region. This preliminary finding of identification of *P. oryzae* USM-PD1 will be subjected to further study for the biological control of rice blast disease. The present study provides information on the etiology and symptomatology of rice blast disease caused by *P. oryzae*. Further research would include studies on multigene variation and rice blast disease management.

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CONFLICTS OF INTEREST

All authors declare that there is no conflict of interest.

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