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Molecular Detection of Rust Fungus (*Puccinia* spp.) on Sugarcane using ITS region

Ahmad Ilham Tanzil[1*], Dinda Mala Maulida[1], Wahyu Indra Duwi Fanata[2], Intan Ria Neliana[3], Bambang Sugiharto[4], Cassidy Magnon[5]

[1]Agrotechnology Study Program, Faculty of Agriculture, University of Jember, Jember 68121, Indonesia
[2]Agricultural Science Study Program, Faculty of Agriculture, University of Jember, Jember 68121, Indonesia
[3]Agricultural Technology Study Program, Faculty of Agriculture Technology, University of Jember, Jember 68121, Indonesia
[4]Biology Study Program, Faculty of Mathematics and Natural Science, University of Jember, Jember 68121, Indonesia
[5]LSUAg Center, Louisiana State University, Baton Rouge 70803, LA, United States of America

*Corresponding Author: aitanzil@unej.ac.id

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Abstract. Identifying pathogenic fungi at the species level based on morphology, especially for leaf rust, is quite difficult. Further investigation, such as molecular techniques, is required to identify leaf rust fungi precisely. One way that can be employed is DNA barcoding. This study seeks to detect leaf rust fungi (*Puccinia* spp.) on sugarcane. Accurate species identification allows early detection of Puccinia kuehnii, helping farmers and extension agencies to apply targeted control strategies. A fungal sample was collected from sugarcane fields in Bondowoso, East Java, Indonesia. Morphological identification was done with an SEM, whereas molecular identification was done through DNA extraction, amplification, visualization, sequencing, and phylogenetic tree creation. The primers employed are obtained from the ITS region of ribosomal DNA, which contains enough diversity to differentiate fungi at the species level. The amplification findings revealed a DNA band of 500 bp at both loci. Sequencing results indicate that samples The sequencing results indicatethat the samples from Bondowoso are closely related to the *Puccinia kuehnii* sequence from NCBI. The findings of the phylogenetic tree construction revealed that samples from both locations are still associated with *P. kuehnii* sequences from other nations. This is the first study documenting *P. kuehnii* in Indonesia, especially East Java, that integrates morphological and molecular characterization.

Keywords: Bioinformatic; ITS Region; ribosomal DNA; NCBI (National Center for Biotechnology Information); SEM (Scanning Electron Microscopy)

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INTRODUCTION

Sugarcane rust is a disease that infects sugarcane plants and other species within the Poaceae family. This disease is caused by fungi of the genus Puccinia (Kjellström, 2021). In fact, sugarcane rust is considered one of the major diseases in sugarcane cultivation in several countries, including Florida, United States (Sanjel et al., 2019). The impact of fungal infection includes reduced crop productivity, with the severity depending on the cultivar and environmental conditions. Sugarcane is an important crop for the sugar and bioethanol industries in Indonesia. Detecting leaf rust fungi at an early stage helps reduce yield losses and supports national food and energy security. To date, three species of sugarcane rust fungi have been reported: *Puccinia kuehnii* (the causal agent of orange rust), *Puccinia melanocephala* (brown rust), and *Macruropyxis fulva* (tawny rust) (Martin et al., 2017).

Initially, sugarcane rust fungi were not regarded as major pathogens and were considered relatively harmless. However, their status changed following reports of severe infections on several widely cultivated commercial sugarcane varieties, such as B 4362. This particular variety was eventually withdrawn from cultivation in several countries due to susceptibility to *P.*

melanocephala (Magarey, 2022). B 4362 is recognized as the most vulnerable variety, showing significant yield losses wherever it is grown. In Indonesia, several sugarcane varieties, including PS881, PS92-752, and 6535, are reported to be moderately susceptible to *P. kuehnii* (Ismayanti & Hadisutrisno, 2013)(Neliana et al., 2024). So far, only two rust species have been reported in Indonesia: *P. melanocephala* (brown rust) and *P. kuehnii* (orange rust).

Comprehensive knowledge of sugarcane rust fungi, including their identification based on morphological and behavioral characteristics, is essential for effective disease management. However, morphological identification is often insufficient due to the difficulty in distinguishing (Manzar et al., 2022) among rust species based on physical characteristics alone. Furthermore, fungal growth is influenced by sugarcane variety, climate, and regional environmental conditions (Glynn et al., 2010)(Montes et al., 2021), suggesting the possibility of location-specific fungal diversity. Misidentification has also been common in previous studies, particularly within the Pucciniaceae family, as some rust fungi exhibit highly similar morphologies (Yadav et al., 2023) and can only be accurately distinguished through molecular identification (Roy et al., 2018). For instance, *P. kuehnii* is frequently misidentified as *P. melanocephala* or *Macruropyxis fulva* (Martin et al., 2015). In Indonesia, research on sugarcane rust fungi remains limited, emphasizing the need for molecular-level identification.

Molecular characterization of sugarcane rust fungi can be achieved using DNA barcoding. This method includes sample collection, DNA extraction, Polymerase Chain Reaction (PCR), sequencing, and comparison of the resulting sequences with reference databases to determine species-level identity (Keatley et al., 2020). The Internal Transcribed Spacer (ITS) region is commonly used in this process (Harnelly et al., 2022), as it does not encode functional proteins and therefore exhibits higher mutation rates and variability compared to coding regions such as the Small Ribosomal Subunit (SSU) and Large Subunit (LSU) (Paloi et al., 2022). The high genetic diversity of ITS across species (Zhu et al., 2023) makes it a valuable marker in phylogenetic and evolutionary studies (Choi et al., 2019) (Gautam, 2021).

This research advances the understanding of *Puccinia* spp. in tropical sugarcane plantations by refining their taxonomic classification and phylogenetic resolution. As rust fungi, *Puccinia* spp. may play a role in the natural regulation of plant-parasitic fungi. Their responsiveness to environmental fluctuations underscores their significance as pathogens of sugarcane leaves. The findings contribute to sustainable agricultural practices through improved epidemiological management and reduced reliance on chemical inputs. Moreover, this study establishes a foundation for future ecological monitoring and conservation efforts in Indonesia. To accurately identify Puccinia species infecting sugarcane in Bondowoso, East Java, using a combination of morphological (SEM) and molecular (DNA barcoding with ITS region) approaches, overcoming the limitations of morphology-based identification.

METHODS

Rust Fungus Sampling

The samples were collected using purposive sampling, by selecting sugarcane leaves showing symptoms of sugarcane rust fungus, and also healthy leaves without symptoms. Sampling was carried out from May to June 2024 during the dry season at Bondowoso Regency (BWS). Rust samples were taken from the lower leaves of 9-month-old sugarcane plants. The samples were then placed in a cool box and transported to the Molecular Biology and Biotechnology Laboratory, Waste Management Unit, and Integrated Laboratory, University of Jember.

SEM Analysis

SEM observation was carried out by preparing the SEM equipment and the sample stage. Then, the selected part of the leaf to be observed was chosen. The designated leaf part was attached to carbon tape. The carbon tape was placed on the sample stage, which was then mounted onto the holder inside the chamber. Next, a low vacuum of 90 Pa was applied, and the voltage was set to 5 kV. The spot size was adjusted to 2.5, and brightness and contrast were set as needed. Finally, the sample was scanned at 10 μS (microsiemens) (Erdman et al., 2019).

DNA Extraction

DNA extraction of the pathogenic fungus was carried out using the SDS method. A total of 0.25 grams of symptomatic sugarcane leaf samples were weighed and placed in liquid nitrogen. Each sample was ground finely using liquid nitrogen in a mortar and transferred into an extraction buffer containing 600 μl and 30% SDS. The samples

were then vortexed and incubated at 65°C for 10 minutes. After incubation, 600 µl of PCI was added, followed by vortexing and centrifugation at 12,000 rpm for 10 minutes at room temperature.

The supernatant was collected and mixed with 600 µl of isopropanol, then inverted until homogeneous. The mixture was centrifuged again at 12,000 rpm for 10 minutes at room temperature, and the supernatant was discarded. In the next step, 50 µl of TE buffer and 2 µl of RNase were added, then incubated at 37°C for 60 minutes. After that, 500 µl of TE buffer and 500 µl of PCI were added to the sample, vortexed, and centrifuged at 12,000 rpm for 10 minutes at room temperature. A total of 500 µl of the supernatant was then transferred to a new tube. Next, 400 µl of isopropanol and 100 µl of sodium acetate were added, and the mixture was inverted until homogeneous. The sample was centrifuged at 12,000 rpm for 10 minutes at room temperature and then incubated at -20°C for 60 minutes.

After incubation, the sample was centrifuged again at 12,000 rpm for 10 minutes at room temperature, and the supernatant was discarded. The sample was then washed with 70% ethanol and centrifuged at 12,000 rpm for 10 minutes at 4°C. This ethanol washing step was repeated, and finally, the DNA pellet was incubated in an oven at 45°C for one hour and resuspended in 30 µl of TE buffer (Tanzil & Fanata, 2024).

PCR Amplification of the ITS Region

Amplification of the pathogenic fungus was carried out using specific primers for *Puccinia* spp., namely PK-F: (5'-GGA GTG CAC TTA ATT GTG GCT C-3') and PK-R: (5'-GCA GGT AAC ACC TTC CTT GAT GTG-3') (custom-synthesized), which target the ITS1 and ITS2 regions of *Puccinia* spp. The PCR reaction had a total volume of 15 µL, consisting of 7.5 µL of Go Taq® Green Master Mix (Promega), 1 µL of PK-F primer and 1 µL of PK-R primer (100 µM), 1 µL of DNA template, and 4.5 µL of nuclease-free water.

The PCR machine used was a Bio-Rad T-100 Thermal Cycler with 35 cycles under the following conditions: initial denaturation at 95°C for 5 minutes; denaturation at 95°C for 30 seconds; primer annealing at 55°C for 30 seconds; extension at 72°C for 1 minute; and a final extension at 72°C for 7 minutes. The PCR products were electrophoresed on 1% agarose gel in TAE buffer and visualized using a GelDoc system (Major Science) (Sánchez-Flores et al., 2025).

Sequencing and Phylogenetic Tree Reconstruction

Sequencing was performed by sending the amplified DNA fragments to PT. Genetika Science Indonesia. The sequencing results were analyzed using the BioEdit software and compared with sequences in the GenBank NCBI database through the BLAST (Basic Local Alignment Search Tool) program. The genetic relationship among the sequences and the construction of the phylogenetic tree were carried out using the UPGMA (Unweighted Pair Group Method with Arithmetic Mean) method in the MEGA11 software (Pradana & Yoshiga, 2025).

RESULTS AND DISCUSSION

Diseases Symptoms

Sugarcane rust wilt disease was found in the exploration site. Sugarcane plants infected by rust disease showed symptoms characterized by yellow spots that gradually turned reddish-brown. In addition, urediniospores were observed on the underside of the leaves during the late stage of infection (Figure 1). Urediniospores emerged through ruptured leaf tissue (pustules). In severe infections, pustules spread to other parts of the leaf. Open pustules revealed urediniospores that varied in color, shape, and size. The color of pustules is used as an indicator to identify the rust-causing pathogen. Orange-colored pustules are a typical symptom of *Puccinia kuehnii*, while brown-colored pustules are characteristic of *Puccinia melanocephala* (Selvakumar & Vismawathan, 2019). However, early rust symptoms can resemble those of yellow or brown leaf spots, which are not caused by *Puccinia* spp. Therefore, accurate identification of rust type requires observation of advanced symptoms. The color and shape of pustules are commonly used to differentiate rust species (Rott et al., 2023). For accurate diagnosis, fully developed pustules must be examined, as early symptoms of different rusts are very similar. Orange pustules are a typical sign of *P. kuehnii* (Perera et al., 2020a), while brown pustules indicate P. melanocephala infection (Chen et al., 2025).

Morphological Observation

The fungal morphology observed was that of urediniospores, which appear during the late stage of infection. Based on morphological analysis using a scanning electron microscope (SEM), samples from Bondowoso Regency (BWS) showed urediniospores with ellipsoidal and

obovoid shapes (Figure 2). The color of the urediniospores, as seen from the symptoms on infected sugarcane leaves (Figure 1), ranged from orange to dark brown. The surface of the spores was found to have spines (spine/echinulation). *Puccinia* spp. are difficult to distinguish at the species level due to their similar morphological characteristics. The symptoms of sugarcane rust are visually distinctive, making the disease relatively easy to identify in the field. In susceptible varieties, pustules appear on the leaves (Sopialena et al., 2022). These pustules develop when asexual spores (urediniospores) germinate on the leaf surface and grow in colonies within the leaf tissue (Kayim et al., 2022). As the spores are continuously produced, the leaf tissue becomes torn, releasing new spores and repeating the infection cycle (Garnica et al., 2014). The presence of pustules can reduce the photosynthetic rate and chlorophyll content, as observed in other rust infections such as *P. coronata* on oats (Sowa & Paczos-GrzeRda, 2021) and *P. triticina* on wheat (Kolmer et al., 2022).

Figure 1. Symptom of sugarcane leaf rust (Left); Healthy leaf (Right)

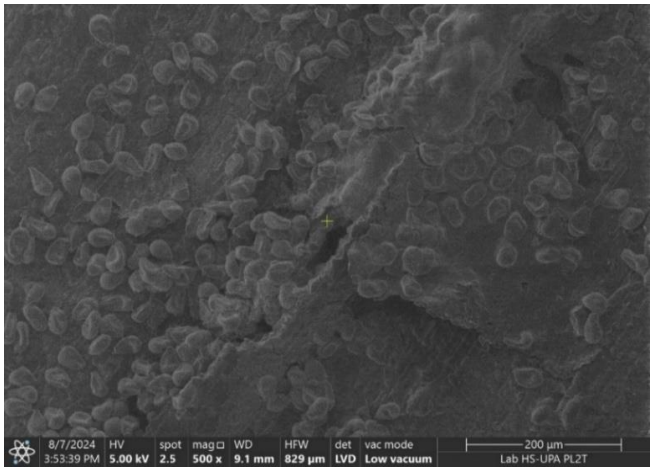

Figure 2. Spore Morphology of Puccinia spp.

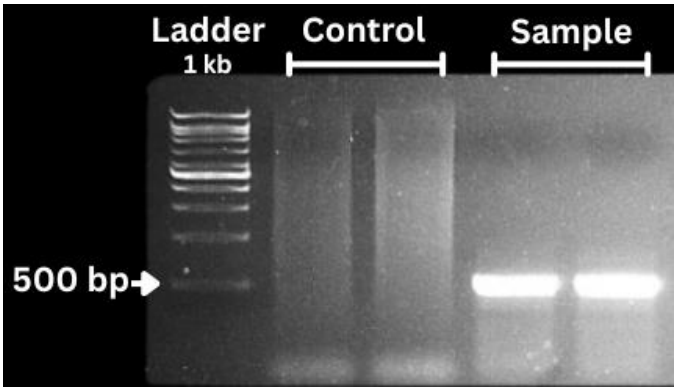

Figure 3. DNA amplification results of Puccinia spp.

Molecular Identification

The PCR amplification of DNA from BWS samples using the PK-F and PK-R primers showed a DNA band of approximately 500 bp (Figure 3), indicating that the observed pathogenic fungus belongs to the *Puccinia* genus. The sequences obtained in this study were registered with GenBank with accession number PQ799505. Sequence analysis of the BWS sample revealed low genetic diversity, which is attributed to their high homology levels (Table 1). The genetic distance of Puccinia spp. from both BWS samples was less than 0.1, indicating relatively low genetic variation among *Puccinia* species (Figure 4). Based on molecular analysis, the rust fungi observed in both Bondowoso were identified as *Puccinia kuehnii*, due to their high sequence homogeneity with *P. kuehnii* in the NCBI database. Thus, the sugarcane rust disease found in the location is classified as orange rust. Among the two surveyed sites, the Bululawang (BL) variety was most frequently found infected by orange rust. This indicates that the Bululawang variety tends to be susceptible to *P. kuehnii*, although previous research (Ismayanti & Bambang Hadisutrisno, 2013) categorized it as moderately resistant to this pathogen.

Table 1. The level of nucleotide and amino acid homology of Puccinia spp. BWS samples

Species	Country	Accession Number	Homology (%)
			Nucleotide
Puccinia kuehnii	Indonesia	PQ799505	100
Puccinia kuehnii	Australia	FJ708562	99
Puccinia emanculata	United States	KX190848	69
Puccinia setariae	United States	KX190907	87
Puccinia chunjiei	China	HQ012446	87
Puccinia novopanici	United States	KX190874	94
Puccinia aizazii	Pakistan	KY767027	87
Trichoderma atroviride	Italy	FJ975598	25

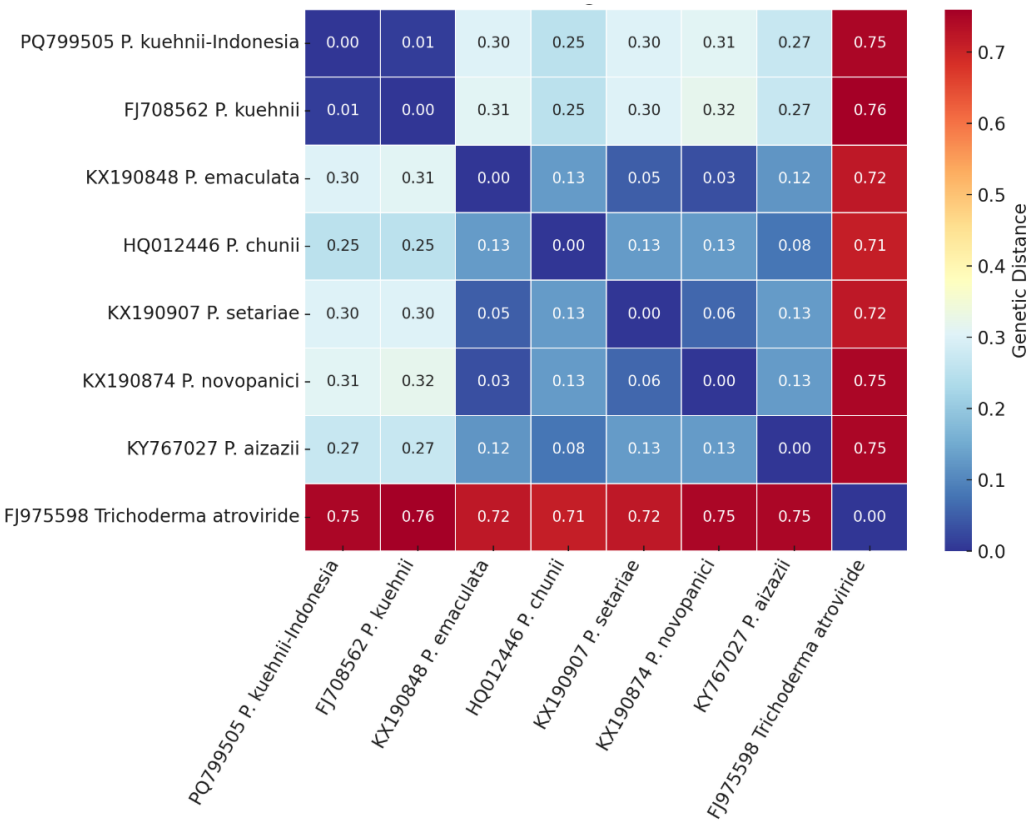

Figure 4. Genetic distance of Puccinia spp. BWS samples with sequences in GenBank

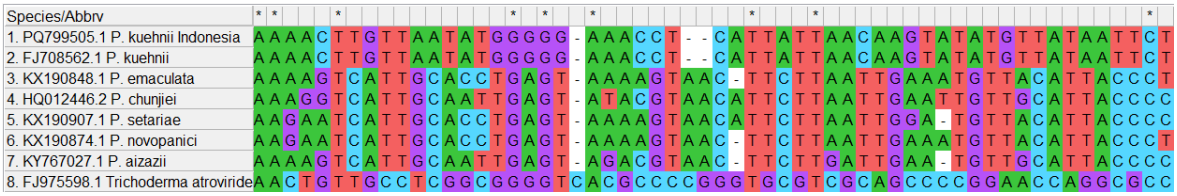

Figure 5. BWS sequence alignment. BWS samples show nucleotide base variations with the Puccinia spp. sequence from NCBI

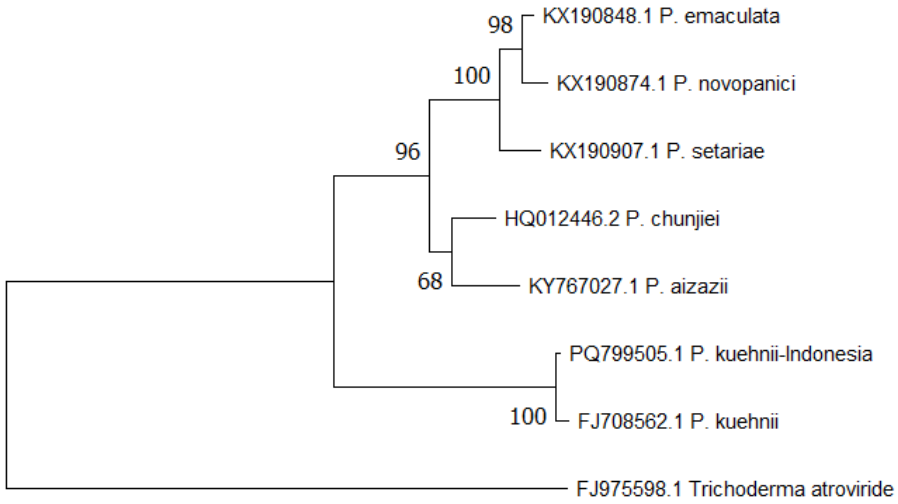

Figure 6. Phylogenetic tree of Puccinia kuehnii in Sugarcane at Bondowoso Regency, Indonesia, along with other Puccinia sequences from GenBank. The tree was constructed using MEGA v11.0.10 with the neighbor-joining method and 1,000 bootstrap rep

This result is supported by the presence of nucleotide variations observed in the alignment of BWS sequences using BioEdit software (Figure 5). These sequencing results are consistent with the phylogenetic tree analysis. The phylogenetic analysis showed that the *Puccinia* spp. sample from Bondowoso Regency clustered with *Puccinia kuehnii* sequences from Indonesia, with a bootstrap value of 100% (Figure 6). Genetic distance analysis using MEGA11 revealed that the ITS region has low genetic diversity due to the presence of highly conserved areas. According to (Bradshaw et al., 2023) and (Ciardo et al., 2006), ITS sequences from eight fungal genera show very high base similarity (homology), with several reaching 67%–100%. Although the ITS region contains highly conserved sequences, it still has significant variation between species due to frequent mutations (Mulyantni et al., 2011)(Mulyantni et al., 2011. Despite its conserved nature, the ITS region is widely used for fungal species identification, as more than 70% of ITS-based classifications are accurate at the genus or species level (Porter & Brian Golding, 2011(Porter & Brian Golding, 2011). Because early symptoms and urediospore morphology of sugarcane rust diseases are very similar, they can be difficult to differentiate without expert knowledge. Therefore, molecular identification is necessary to confirm the causal fungus at the species level. The use of Puccinia-specific primers designed based on the ITS region has proven effective in amplifying target DNA, even when extracted together with plant tissue. The application of DNA barcoding successfully detected the sugarcane rust pathogens in Bondowoso as P. kuehnii, confirming it as the causal agent of orange rust disease.

The novelty of this study lies in its position as the first molecular confirmation of *Puccinia kuehnii* (orange rust) infecting sugarcane in Bondowoso, East Java, Indonesia. Unlike previous research in the country, which primarily relied on morphological descriptions that are often prone to misidentification, this study integrates Scanning Electron Microscopy (SEM) with DNA barcoding of the ITS region to achieve species-level identification. The use of both approaches provides a more accurate diagnostic framework and reduces the possibility of taxonomic errors. Furthermore, by comparing local isolates with global sequences available in GenBank, this study contributes valuable molecular data to the global phylogenetic understanding of *Puccinia* spp. and

highlights the genetic similarity of Indonesian isolates with those reported in other sugarcane-producing regions.

Future research should build upon these findings in several ways. First, employing multi-gene markers or whole-genome sequencing would strengthen taxonomic resolution beyond ITS-based identification. Second, pathogenicity and virulence assays across different sugarcane varieties in Indonesia are needed to clarify the host–pathogen relationship and support the development of resistant cultivars. Third, long-term epidemiological monitoring is recommended to assess the spread, seasonal dynamics, and environmental factors influencing *P. kuehnii* outbreaks under changing climatic conditions. In addition, molecular studies focusing on host–pathogen interactions through transcriptomic or proteomic approaches may provide insight into resistance mechanisms and inform sustainable control strategies. Finally, population genetics and phylogeographic studies comparing isolates from different regions will help to track pathogen migration and evolutionary patterns. Together, these directions will deepen the understanding of *P. kuehnii* biology, support sustainable sugarcane disease management, and contribute to the broader field of plant pathology in tropical agriculture**.**

CONCLUSION

The morphological (SEM) analysis identified the rust fungus from the leaves of sugarcane plants in Bondowoso regency, Indonesia, as *P. kuehnii.* and the rDNA sequence of the rust fungus was determined. Further studies, including both morphological and molecular analyses, are necessary to infer the phylogenetic relationship among the *Puccinia* species. Future research should explore multi-gene or whole-genome approaches, pathogenicity tests on different sugarcane cultivars, and long-term epidemiological monitoring to better understand the biology and spread of Puccinia kuehnii.

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AUTHOR CONTRIBUTION STATEMENT

All authors made substantial contributions to the development of this manuscript. AIT and IRN conceptualized and designed the study, while AIT performed the data analysis. Data collection was carried out by DMM and AIT, with CM providing support in the literature review. IRN and DMM contributed to data visualization through analytical tools, and WIDF, together with BS, offered critical feedback during the manuscript drafting. The initial draft of the article was prepared by AIT and DMM. All authors reviewed and approved the final version of the manuscript and agreed to take responsibility for its content in its entirety.

CONFLICT OF INTEREST STATEMENT

The authors declare that there is no conflict of interest regarding the publication of this paper.

USE OF ARTIFICIAL INTELLIGENCE (AI)-ASSISTED TECHNOLOGY

The authors declare that no artificial intelligence (AI) tools were used in the generation, analysis, or writing of this manuscript. All aspects of the research, including data collection and manuscript preparation, were carried out entirely by the authors without the assistance of AI-based technologies. Only Figure 4, we use Chat GPT 4.0 to help in beautifying the image in data interpretation.

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