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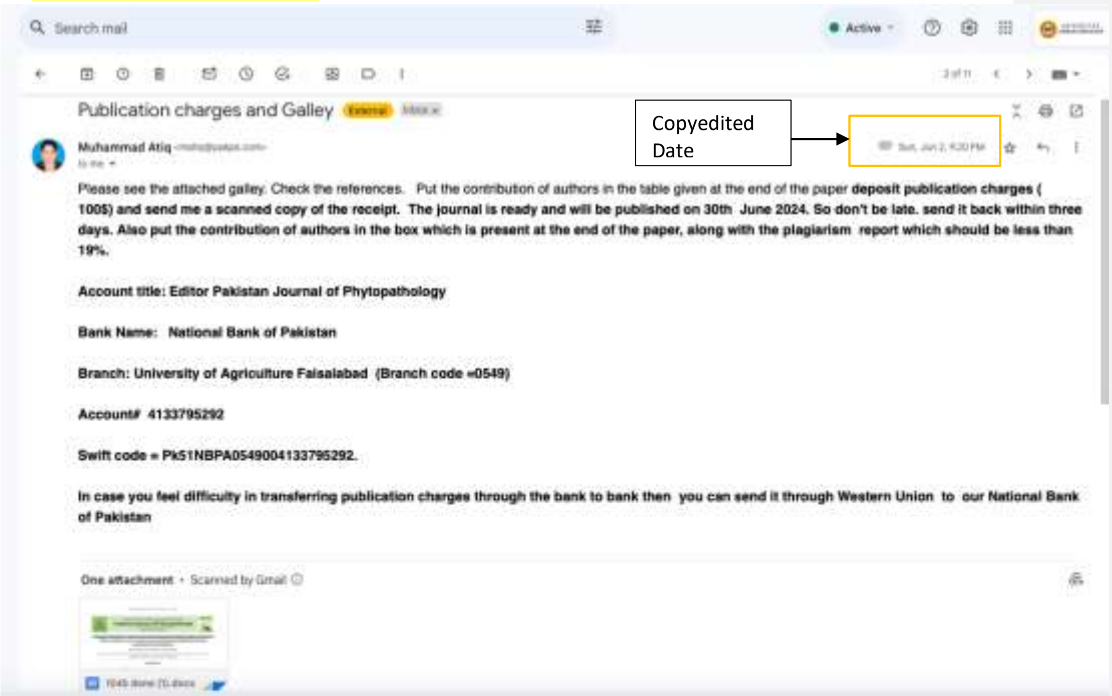

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GENETIC DIVERSITY AND NUCLEOTIDE SEQUENCE CHARACTERIZATION OF THE P4 GENE OF RICE TUNGRO BACILLIFORM VIRUS (RTBV) IN SOUTH KALIMANTAN, INDONESIA

Noor Aidawati, Saipul Abbas, Elly Liestiany

Abstract

Rice tungro bacilliform virus (RTBV) is a serious threat to rice production in various regions in Indonesia including South Kalimantan. Cautious and flexible management strategies are of utmost importance. This study investigates the genetic diversity within the P4 gene of *RTBV* in South Kalimantan, Indonesia, to inform targeted disease management strategies. The PCR method was used to amplify P4 gene fragments from *RTBV* samples collected from 2 locations, namely *Karang Buah* Village and *Anjir Pasar Lama*. The PCR results successfully detected fragments of 430 bp, which were then analyzed in sequence to identify genetic variations. The characterization results showed that the presence of significant genetic diversity among *RTBV* isolates. These variants include differences in nucleotides at specific positions in the P4 gene, indicating the adaptation of the virus to local conditions. These findings provide a solid basis for designing more precise control strategies, such as the development of rice varieties that are more resistant to certain *RTBV* variants or the implementation of more specific management measures.

Keywords

PCR; P4 Gene; RTBV; Rice; Sequence Characterization

KEYWORDS

Begomovirus Botanicals Chickpea Cultivars FHB species Fungicides Fusarium oxysporum Isolation Petri-dish assay Plant extracts Pyricularia oryzae Rice Wheat biological efficiency chickpea disease incidence freezing morphology viability water storage yield

First revision

PCR Analysis and Nucleotide Sequence Characterization of gene P4 to control Tungro Viruses (RTBV)

Noor Aidawati[a], Saipul Abbas[b*], Elly Liestiany[c]

[abc]Department of Plant Protection, Faculty of Agriculture, Lambung Mangkurat University. Jl.Jend. A. Yani Km.36 Banjarbaru. South Kalimantan 70714 Indonesia
[*]Email: saipul.abbas@ulm.ac.id

ABSTRACT

Rice tungro bacilliform virus (RTBV) is a serious threat to rice production in various regions in Indonesia including South Kalimantan, so it is necessary to take a careful and adaptive control approach. This study aims to conduct PCR analysis and nucleotide sequence characterization in the P4 RTBV gene as a basis for formulating a more precise control strategy. The PCR method was used to amplify P4 gene fragments from RTBV samples collected from 2 locations, namely *Karang Buah* Village and *Anjir Pasar Lama*. The PCR results successfully detected fragments of 430 bp, which were then analyzed in sequence to identify genetic variations. The characterization results showed that the presence of significant genetic diversity among RTBV isolates. These variants include differences in nucleotides at specific positions in the P4 gene, indicating the adaptation of the virus to local conditions. These findings provide a solid basis for designing more precise control strategies, such as the development of rice varieties that are more resistant to certain RTBV variants or the implementation of more specific management measures.

Keywords: PCR, P4 Gene, Nucleotide Sequence Characterization, RTBV, Control Strategy.

Comment [AR1]: Management strategies will be better.

Comment [DR2]: Rephrase this sentence

Introduction

Rice tungro bacilliform virus (RTBV) is a virus that attacks rice plants (*Oryza sativa*) and causes tungro disease. RTBV belongs to the family *Caulimoviridae* and the genus *Badnavirus* (Bömer et al., 2018). The virus has a single-stranded DNA-shaped genome and is generally transmitted via insect vectors. The transmission of RTBV by the leafhopper *Nephotettix vitrescens* is dependent upon the *Rice tungro spherical virus* (RTSV). The transmission of RTBV and RTSV is semipersistent in adult leafhoppers (Hibino & Cabauatan, 1987). In addition to an eye examination, scientific confirmation of the condition is currently achieved through the use of polymerase chain reaction (PCR) technologies that detect viral nucleic acids (Yee et al., 2017). The PCR method is simpler and quicker than other methods, it is highly beneficial for identifying the presence of rice viruses (Abbas et al., 2020). PCR analysis was used to identify and amplify the P4 gene of RTBV, allowing detection of the presence of the virus in the sample. P3 and P4 gene amplification was done using PCR (Pouraziz et al., 2023).

Comment [DR3]: Made primers from the reported sequences

Characterization of the nucleotide sequence of the P4 RTBV gene involves determining the nucleic acid sequence, thus providing an understanding of the structure and genetic variation of the virus. Reduced 21-nt siRNA levels and increased 22-nt siRNA levels were linked with RTBV protein P4's suppression of cell-to-cell spread of silencing and enhancement of cell-autonomous silencing (Rajeswaran et al., 2014). The strategy of tungro disease control in rice plants is strengthened through the genetic understanding of RTBV, resulting from PCR analysis and P4 gene sequencing. A key component of disease control strategies is identification, which is one of the initial phases (Afandi et al., 2022). *Rice tungro bacilliform virus* (RTBV) was the focus of research to identify genetic variations, opening up the potential for the development of more adaptive control methods. A deep understanding of the genetics of RTBV through PCR analysis and characterization of P4 gene sequences became the foundation for designing more effective control strategies in increasing the resistance of rice plants to viral attacks.

Although PCR analysis was able to identify the P4 RTBV gene, it remains unclear to what extent this genetic variation influences the virulence and spread of the virus. Vector is usually linked to the unintentional consequences of viral infection on host quality, which might increase virus dissemination. The host plant's secondary chemistry can be altered by viruses, which can have an impact on the behavior of vectors (Chisholm et al., 2019; Ingwell et al., 2012) . It is not yet known exactly how nucleotide variations in the P4 RTBV gene can affect the response of rice plants to infection, as well as the extent of adaptation of the virus to different plant varieties. Nucleotide variation-causing positions were taken out of the alignment. Three types of sites were found to be potentially beneficial for studying substitution frequency, at which only two specific residues occurred. Those where a specific residue was found in a single isolate were split into two categories (Cabauatan et al., 1999). However, a total of 28 Single Nucleotide Variations (SNVs) were discovered (Reen et al., 2019). The influence of RTBV genetic diversity on viral replication mechanisms and how it interacts with its host is still a question that needs to be answered through further research. The susceptibility of different rice varieties to RTBV variants is still not fully understood, so further research is needed to assess and compare the level of resistance or sensitivity of certain rice genotypes to the virus.

This research is important to understand more deeply about the *Rice tungro bacilliform virus* (RTBV). Through a genetic understanding of RTBV, more effective control strategies can be formulated, such as the selection of rice varieties resistant to specific variants and the implementation of targeted control methods. This is important to improve the sustainability of rice farming and reduce the impact of tungro disease on crop yields. This study aimed to uncover patterns of genetic diversity that might influence the virulence and adaptation of viruses to the environment as well as rice plant varieties. In addition, this research is directed to formulate more effective control strategies, including the selection of rice varieties that are resistant to certain variants and the application of targeted control methods.

Research Methodology

Sampling Location

Sample collection was carried out in two locations, namely in *Karang Buah* Village and *Anjir Pasar Lama* Barito Kuala Regency, South Kalimantan. Samples of rice plants that showed typical symptoms of RTBV infection, such as yellowing and curling leaves, were selected for further analysis. These samples were collected using field investigation methods involving surveys and direct observation by the research team.

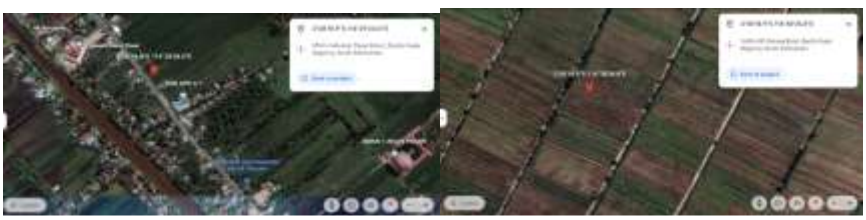

Figure 1. Map of the sampling location of symptomatic rice plants infected with tungro virus like symptoms. (a) *Anjir Pasar Lama* Village, (b) *Karang Buah* Village, South Kalimantan Indonesia

DNA amplification by PCR

DNA isolation was performed using a total DNA extraction kit (GeneAid) from infected rice plant samples. Plant samples were cleaned to free plant cells. DNA extraction was performed using a standardized extraction protocol. The primary design was carried out based on the previously known nucleotide sequence of the P4 RTBV gene. The selected primers were directed to amplify P4 gene fragments with a length of about 430 bp. The PCR reaction was carried out in a PCR reaction body tube containing the master mix reaction solution, RTBV-B2F/B2R primers, and sample DNA. PCR cycles were arranged according to an optimized thermal profile to ensure target amplification which can be seen in the following table.

Table 1. PCR Procedure Parameters

Component	Volume (µl)	PCR Program	Temperature (º C)	Time	Cycle
PCR mix	5	Initial denaturation	95	2	1
RTBV-B2F primer	1	Denaturation	95	30”	35
RTBV-B2R primer	1	Annealing	53	30”	35
Nuclease Free Water	4	Extension	72	30”	35
DNA	1	Final extension	72	7’	1
		Stop	4	∞	-

Table 2. Primers used during PCR

Primer	Primer sequence	gene	DNA Fragment size (bp)	annealing temperature (^{o}C)	Reference
RTBV-B2F	B2-F: 5-GCAGAACAGAACTCTAAGGC-3				
RTBV-B2R	:"5-GTCTAAGGCTCATGCTGGAT-3	P4	430	53	(Cabauatan et al., 1999)

The resulting PCR products were analyzed using 2% agarose electrophoresis to check the amplification success and detect the expected P4 gene fragment size. Agarose gel was prepared, and PCR products were loaded in gel wells. Electrophoresis was performed using a constant electric current to

Comment [DR4]: Not mentioned in the text

Comment [DR5]: Rephrase.

Comment [DR6]: This is mixing of two tables into one. Either give volumes or the timings for the cycle

Comment [DR7]: Use appropriate sign

Comment [DR8]: What is this for?

Comment [DR9]: Please verify

separate DNA fragments by size. After electrophoresis, the gel was visualized using *ethidium bromide*DNA dye and visualized using UV light to see the DNA bands.

Sequencing DNA

Successfully amplified and verified PCR products were then purified using DNA purification methods to remove contaminants and residues of PCR reagents. The purified DNA was then submitted for nucleotide sequences using Sanger sequencing technology at a trusted nucleotide sequence center (PT. Genetics Science Indonesia). The nucleotide sequence data obtained were further analyzed to obtain information about genetic variation in the P4 RTBV gene.

The nucleotide sequence data obtained were analyzed using MEGA XI software. The BLAST NCBI bioinformatics analysis tool was used to compare the nucleotide sequence of PCR results with the RTBV nucleotide sequence reference in the GenBank database. Phylogenetics analysis and dendrogram percentage homology algorithm was created using Mega XI software, while the results of Multiple sequence alignment using Clustal W (EMBL-Ebi). The results of bioinformatics analysis were used to interpret the genetic diversity of RTBV and identify significant patterns of genetic variability. This information is then used as a basis for formulating more adaptive and specific control strategies.

Results and discussion

Visual Symptoms

Based on observations in two locations, namely *Anjir Pasar Lama* Village and *Karang Buah*, some symptoms indicate tungro virus infection Plant leaves showed signs of yellowing, which was an indication of symptoms of tungro disease. Plants also showed symptoms of stunting In addition, green leafhopper vector insects were found which are carriers of tungro virus. The presence of green leafhoppers can be a factor in the spread of the disease.

Figure 2. Visual Symptoms of Tungro Disease Observed in the Field.

Comment [DR10]: Again not mentioned in the text

The interaction between rice plants, tungro virus, and green leafhoppers as vectors needs to be better understood. Further research can focus on the ecological dynamics of green leafhoppers, including their movement patterns and behavior in agricultural ecosystems. Insect migrations are defined generically as the seasonal movements of insect populations involving vast numbers of individuals that repeat in terms of location (across the same geographic area), time (annually or otherwise), and direction (Satterfield et al., 2020).This further information can help formulate more effective and sustainable control strategies. Related to social impacts, the spread of tungro virus can also trigger food crises at the local and regional levels. Devastating losses in total crop production or produce quality can occur when a large number of plants contract a systemic virus infection and the infection results in severe disease symptoms (Jones, 2021). Furthermore, massive epidemics or pandemics of virus-related diseases that affect staple food crops that are vital to food security have the potential to drastically reduce food supplies, leading to famine in the event of severe shortages (Jones, 2014; Jones & Naidu, 2019). Therefore, there needs to be collaboration between farmers, government, and agricultural experts to implement effective preventive measures and provide technical assistance to farmers in facing these challenges. In addition, public education about safe and sustainable agricultural practices can also be key in reducing the risk of spreading this disease in the future

Comment [DR11]: I think this is just a discussion, its not the part of results

PCR Analysis Results

PCR amplification results using a pair of RTBV-B2F/B2R primers specifically designed to amplify DNA fragments from the P4 RTBV were analyzed by agarose electrophoresis to identify the presence of RTBV virus in two site samples. The electrophoresis results showed that PCR successfully amplified 430 bp of P4 gene fragments in all samples tested. The measurement of band intensity in electrophoresis also illustrates the variability in the number of copies of the P4 gene among different isolates.

Comment [DR12]: It should be M for marker

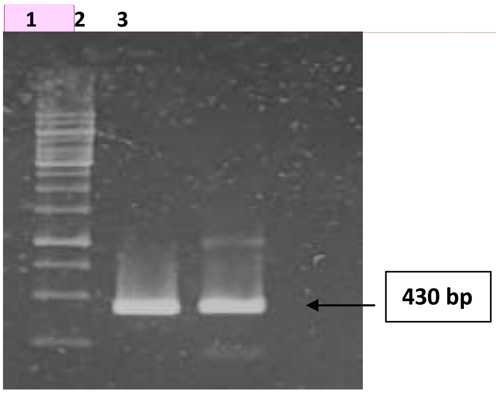

Figure 3. PCR amplification results using a pair of RTBV-B2F/B2R gene P4 primers. (1) Marker 1 kb DNA Ladder; (2) *Anjir Pasar Lama*, (3) *Karang Buah*

Comment [DR13]: Again Not mentioned in text

Comment [DR14]: Pic quality is very bad and needs to be more clear and properly labelled.

The successful amplification of the P4 gene provides the basis for in-depth nucleotide sequence analysis. A good sequence result occurs when the PCR result is successfully amplified with the primer (Green et al., 2015). The genetic diversity detected in the PCR results indicates the potential for functional variation in RTBV gene expression at various sites. This variability can impact the biological properties of the virus, including resistance to the environment or certain plant varieties. This general case—also known as genotypic resistance, host resistance, specific resistance, or cultivar resistance—occurs when a plant taxon exhibits genetic polymorphism for susceptibility, meaning that while some genotypes in the same gene pool are susceptible to a given virus, other genotypes exhibit heritable resistance (Kang et al., 2005).The successful PCR results also open up opportunities for further research, such as gene expression analysis (T. Wang et al., 2019) and further understanding of the mechanisms of RTBV adaptation to local selection pressures (Karasov et al., 2020).

Comment [DR15]: Again this is more like a discussion

Nucleotide Sequence Characterization

The nucleotide sequence of the PCR product was successfully obtained and analyzed. Bioinformatics analysis involves comparing the nucleotide sequence of PCR results with the nucleotide sequence of the P4 RTBV gene that has been registered in the previous GenBank database. The results of the analysis showed significant nucleotide variation at several positions in the P4 gene between RTBV isolates from different regions (Table 3).

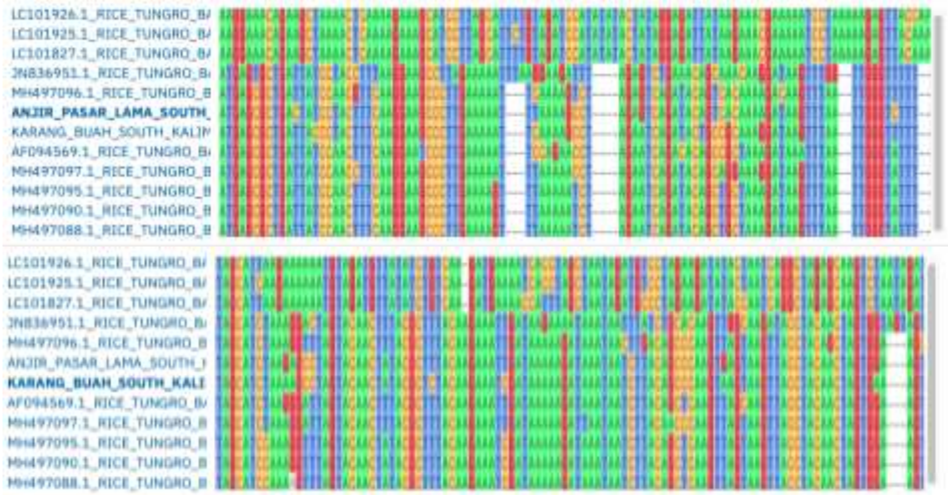

Figure 4. Multiple Sequence Alignment by Clustal W

Comment [DR16]: Not mentioned in text

Comment [DR17]: I think there is something seriously wrong with your alignment as first three are different in behavior than the rest.

Table 3: Nucleotide Variation in the P4 RTBV Gene

Isolate	Isolate nucleotide variations at position
Anjir_Pasar_Lama (S. Kalimantan)	A123G, T456C, G789A
Karang_Buah (S. Kalimantan)	A123G, T456C, G789A
MH497095.1 (Keningau, Malaysia)	A123G, T456C, G789A
MH497090.1 (Kota Belud, Malaysia)	A123G, T456C, G789A
MH497088.1 (Tuaran, Malaysia)	A123G, T456C, G789A
JN836951.1 (India)	A123G, T456C, G789A
AF094569.1 (Thailand)	A123G, T456C, G789A
LC101926.1 (Garut, Indonesia)	A123G, T456C, G789A
LC101925.1 (Sidrap, Indonesia)	A123G, T456C, G789A
LC101827.1 (Bali, Indonesia)	A123G, T456C, G789A

The table provides the results of nucleotide variation at certain positions in the P4 *Rice tungro bacilliform virus* (RTBV) gene for various isolates from various regions, such as Indonesia (South Kalimantan, Garut, Sidrap, Bali), Malaysia (Lundu, Bario, Keningau, Kota Belud, Tuaran), India, and Thailand. Each isolate represents a specific variant of the virus that can have different genetic characteristics. Nucleotide variation shows differences in nucleotides at specific positions in the P4 RTBV gene sequence. This variation is expressed as a change from the expected nucleotide, and this difference is noted as G (Guanine), A (Adenine), T (Thymine), or C (Cytosine) at certain positions in the gene sequence. Example, A123G: Shows that at position 123 in the P4 gene sequence, the nucleotide Adenine (A) in the isolate has changed to Guanine (G). T456C: Indicates that at position 456 in the P4 gene sequence, the nucleotide thymine (T) in the isolate has changed to cytosine (C). G789A: Indicates that at position 789 in the P4 gene sequence, the Guanine nucleotide (G) in the isolate has changed to Adenine (A). Analysis of these nucleotide variations provides information on genetic diversity among RTBV isolates from different regions. An understanding of these variations is important for detailing differences between RTBV variants and can help in designing more specific and effective control strategies, such as the selection of rice varieties that are more resistant to certain variants (Kang et al., 2005) or the development of control methods that can target these specific variants.

Comment [DR18]: Your alignment tells a total different story as there a lot of replacements in alignment

Phylogenetic Analysis

In the phylogenetic analysis provided, we can see a table of homology (%) between samples taken from different locations, which is likely related to RTBV. The homology table shows the percentage of genetic similarity between the samples. The following are the results of phylogenetic analysis and homology percentages for sample comparison based on homology (%) contained in Table 4.

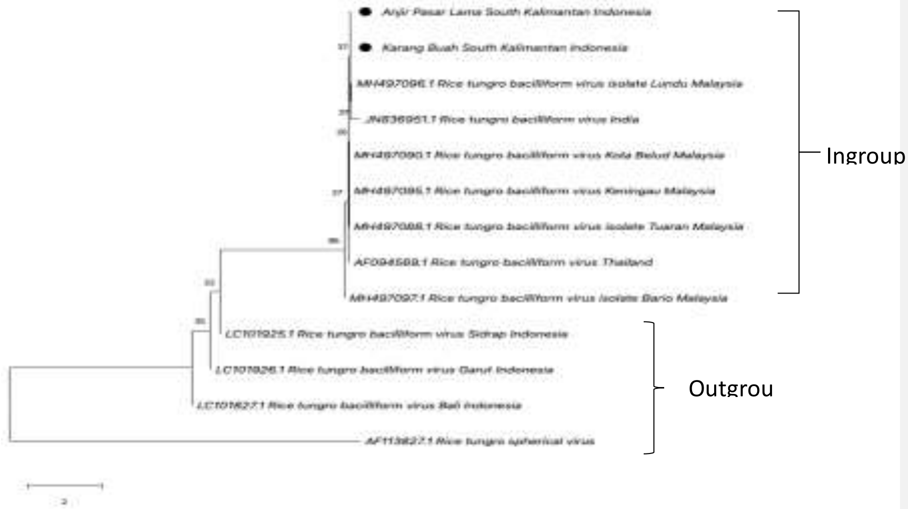

Figure 5. Phylogenetic Tree with bootstrap 1000 replicate

Comment [DR19]: Again not mentioned in the text

Comment [DR20]: The outgroup is only the last one. Your explanation is wrong.

Table 4. Homology percentage value

No.	Sample Origin	Homology (%)							
		1	2	3	4	5	6	7	8
1	Anjir_Pasar_Lama_South_Kalimantan_Indonesia	ID							
2	Karang_Buah_South_Kalimantan_Indonesia	95.05	ID						

3	MH497095.1 RTBV_Keningau-Malaysia	92.76	92.38	ID					
4	MH497090.1 RTBV_Belud-Malaysia	93.11	92.73	99.70	ID				
5	MH497097.1 RTBV_Bario-Malaysia	90.27	90.82	93.39	92.99	ID			
6	MH497096.1 RTBV_Lundu-Malaysia	92.01	90.19	91.12	90.70	89.44	ID		
7	JN836951.1 RTBV_India	72.58	70.34	71.25	71.76	72.18	74.29	ID	
8	AF094569.1 RTBV_Thailand	90.36	88.93	93.09	92.69	91.18	88.09	66.18	ID
9	MH497088.1 RTBV_Tuaran-Malaysia	93.50	93.13	99.70	100.00	92.97	90.67	71.60	92.66

Comment [DR21]: Why this value is so low

Nucleotide sequence analysis of the P4 gene in RTBV Virus offers deep insights into genetic diversity at 2 sites in South Kalimantan. In this context, sequences from South Kalimantan, Indonesia, show distinctive variations, along with isolates from various locations in Malaysia, India, and Thailand. In isolates from South Kalimantan, *Anjir_Pasar_Lama*, and *Karang_Buah*_South_Kalimantan_Indonesia, nucleotide differences at several positions highlight the presence of genetic diversity among these viruses, which may be related to environmental factors or local selection pressures (Kang et al., 2005). Isolates from Malaysia show striking diversity, both in terms of genetic variation between regions such as Keningau, Kota Belud, Tuaran, Bario, and Lundu, as well as in comparison with isolates from Indonesia. These variants may provide clues about RTBV's adaptive response to environmental factors or selection pressures in these different regions.

Comment [DR22]: Which positions? This is your result, focus here

Comment [DR23]: How much diversity

These differences raise interesting questions about how these factors influence the evolution and spread of RTBV. However, the most striking difference may lie in the sequences from India and Thailand, as seen in JN836951.1_*RTBV*_India and AF094569.1_*RTBV*_Thailand. These significant differences signal genetic variation across geographic boundaries, which may be due to geographic isolation, regional selection pressures, or other environmental factors. These results have major implications for understanding patterns of RTBV spread and evolution globally. As an example, when comparing sequences from Indonesia, especially LC101926.1_RTBV_Garut_Indonesia and LC101827.1_RTBV _Bali_Indonesia, with sequences from Malaysia, the identified differences offer valuable insight into intra- and inter-country genetic diversity. Factors such as plant mobility or interaction with disease vectors may contribute to RTBV population dynamics and lead to the formation of genetic variants

Comment [DR24]: Explain that insight

Discussion

More research is needed to assess and compare the susceptibility or sensitivity of certain rice genotypes to the RTBV virus because the susceptibility of different rice varieties to RTBV variants is still not fully understood. In addition to being important transcriptional expression regulators in biological processes, they are most likely also engaged in the vector-induced pathways found in resistant rice cultivars (Y. Wang et al., 2012). The characteristics of virulence adaption demonstrated that care must be taken when creating and implementing resistant rice varieties (Horgan et al., 2017). The results of this study are very important to guide the selection of rice varieties that are more resistant to RTBV attacks, which in turn can improve the overall resilience of rice plants. Less than ten resistance genes were employed in the breeding effort out of the more than 70 resistance genes known to be associated with the rice planthopper, main gene, and quantitative trait loci (QTLs) (Dai et al., 2008). The genes responsible for resistance were found in wild or native rice (Das et al., 2012). Certain native rice cultivars have been found to produce rice grains of high quality while demonstrating resistance and tolerance to biotic and abiotic stresses (Iswanto et al., 2020). More research is needed to answer questions about the influence of RTBV genetic diversity on viral replication mechanisms how viruses interact with their hosts, and how genetic variation affects the ability of viruses to self-replicate and interact with plant cell components. Viral infections modify the intracellular milieu of vulnerable hosts to their advantage by interfering with anti-viral defenses and appropriating processes necessary for the replication of their genomes (Medina-Puche & Lozano-Duran, 2019). How nucleotide variations in the P4 RTBV gene can affect the response of rice plants to viral infections is not yet

Comment [DR25]: Don't start from this

Comment [DR26]: Y looks extra

Comment [DR27]: Your results are very primitive and resistance is very hard to get.

Comment [DR28]: Redundant with results. Put that part here known with certainty. It is also not yet known how adaptive the virus is to various types of plants. Research can explore how RTBV genetic variation contributes to the virus's adaptation to different varieties of rice plants, focusing on genetic changes that allow the virus to overcome the plant's defense system including its effect on plant gene expression. Although PCR can find the P4 RTBV gene, it remains unclear to what extent these genetic variations affect the strength and spread of the virus. Future research can focus on identifying the relationship between nucleotide polymorphisms in the P4 gene and the severity of tungro disease in rice plants.

Conclusion

In the analysis of nucleotide variation, genetic divergence is seen that reflects RTBV adaptation to the environment and different host plant varieties. Despite the variation, nucleotide sequences in the P4 gene remain largely conservative, suggesting the presence of regions that may be vital for viral function. Significant positional polymorphisms were detected, signaling potential differences in virulence and viral spread. This analysis opens up opportunities to identify RTBV virulence variants that could form the basis of more specific control strategies. In addition, the implications of these PCR and sequencing results are particularly relevant for the development of rice varieties that are more resistant to RTBV, with the possibility of selecting genotypes that have superior resistance characteristics. Thus, this deep understanding not only enriches knowledge about the genetics of RTBV but also provides a basis for designing more sophisticated and sustainable control measures to fight tungro disease in rice plants.

Acknowledgement

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Second revision

General Comments:

1. **Title**: Consider adding a bit more specificity to the title. For instance, mention the focus on genetic variation or nucleotide sequencing for a clearer indication of the paper's main focus.
2. **Abstract**:
    - Provide a brief background on the significance of RTBV in Indonesia.
    - Mention the primary findings and their implications more explicitly.
3. **Introduction**:
    - Provide a more structured introduction by clearly stating the research problem, objectives, and the significance of the study.
    - Clarify abbreviations and acronyms when first used (e.g., PCR, RTBV).
4. **Methodology**:
    - **Sampling Location**: Provide details on the sample size and selection criteria.
    - **DNA amplification by PCR**:
        - Include the concentration of the DNA samples used.
        - Clarify any modifications made to the standardized extraction protocol.
    - **Sequencing DNA**:
        - Mention the specific Sanger sequencing method used.
        - Provide details on the quality control measures taken during sequencing.
5. **Results and Discussion**:
    - **Visual Symptoms**: Include photographs or figures illustrating the observed symptoms.
    - **PCR Analysis Results**:
        - Provide statistical analysis to support the observed variations.
        - Discuss the implications of the observed variations in terms of virus virulence and transmission.
    - **Nucleotide Sequence Characterization**:
        - Include a comparative analysis with known sequences in the GenBank.
        - Discuss the potential functional implications of the observed nucleotide variations.
    - **Phylogenetic Analysis**:
        - Provide a detailed interpretation of the phylogenetic tree.
        - Discuss the evolutionary implications of the observed genetic variations.
6. **Discussion**:
    - Provide a more in-depth discussion on the potential mechanisms underlying the observed genetic variations.
    - Discuss the broader implications of the study findings in the context of rice cultivation and disease management.
    - Highlight the limitations of the study and suggest future research directions.
7. **Conclusion**:
    - Summarize the key findings and their significance.
    - Emphasize the practical implications of the study in terms of disease management and rice cultivation.
8. **References**:

- Ensure that all cited references are correctly formatted according to the chosen citation style.
- Include recent and relevant studies to strengthen the literature review.

Additional Suggestions:

- **Figures and Tables**:
  - Ensure that all figures and tables are clearly labeled and referenced in the text.
  - Provide detailed captions explaining the key findings illustrated in the figures and tables.
- **Language and Style**:
  - Ensure consistency in terminology and style throughout the paper.
  - Avoid repetitive phrases and redundancies to improve readability.
- **Ethical Considerations**:
  - Mention any ethical considerations related to sample collection and data analysis.
- **Funding and Acknowledgments**:
  - Clearly acknowledge all sources of funding and support.

Overall, the paper provides valuable insights into the genetic diversity of RTBV in Indonesia. By addressing the above suggestions, you can further enhance the clarity, coherence, and impact of your research.

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GENETIC DIVERSITY AND NUCLEOTIDE SEQUENCE CHARACTERIZATION OF THE P4 GENE OF *RICE TUNGRO BACILLIFORM VIRUS* (RTBV) IN SOUTH KALIMANTAN, INDONESIA

Noor Aidawati*, Saipul Abbas, Elly Liestiany

Department of Plant Protection, Faculty of Agriculture, Lambung Mangkurat University. Jl.Jend. A. Yani Km.36 Banjarbaru. South Kalimantan 70714 Indonesia.

A B S T R A C T

Rice tungro bacilliform virus (RTBV) is a serious threat to rice production in various regions in Indonesia including South Kalimantan. Cautious and flexible management strategies are of utmost importance. This study investigates the genetic diversity within the P4 gene of RTBV in South Kalimantan, Indonesia, to inform targeted disease management strategies. The PCR method was used to amplify P4 gene fragments from RTBV samples collected from 2 locations, namely *Karang Buah* Village and *Anjir Pasar Lama*. The PCR results successfully detected fragments of 430 bp, which were then analyzed in sequence to identify genetic variations. The characterization results showed that the presence of significant genetic diversity among RTBV isolates. These variants include differences in nucleotides at specific positions in the P4 gene, indicating the adaptation of the virus to local conditions. These findings provide a solid basis for designing more precise control strategies, such as the development of rice varieties that are more resistant to certain RTBV variants or the implementation of more specific management measures.

Keywords: PCR; P4 Gene; RTBV; Rice; Sequence Characterization.

INTRODUCTION

Rice tungro bacilliform virus (RTBV), a significant pathogen within the family Caulimoviridae and the genus Badnaviru*s* (Bömer *et al*., 2018), poses a critical threat to rice production in Indonesia, affecting both yield and quality. The virus has a single-stranded DNA-shaped genome and is generally transmitted via insect vectors. The transmission of RTBV by the leafhopper *Nephotettix vitrescens* is dependent upon the *Rice tungro spherical virus* (RTSV). The transmission of RTBV and RTSV is semi-persistent in adult leafhoppers (Hibino and Cabauatan, 1987). In addition to an eye examination, scientific confirmation of the condition is currently achieved through the use of polymerase chain reaction (PCR) technologies that detect viral nucleic acids (Yee *et al*., 2017). The PCR method is simpler and quicker than other methods, it is highly beneficial for identifying the presence of rice viruses (Abbas *et al*., 2020). PCR analysis was used to identify and amplify the P4 gene of RTBV, allowing detection of the presence of the virus in the sample. P3 and P4 gene amplification was done using PCR. The primer sequences used were CP5-5' - ATGAATACGGCCGCGGCTAGAAATC-3' and 5' - CTATTTCGGGTTCTGGACCTGGCA-3' (Khanal and Ali, 2019; Pouraziz *et al*., 2023)

Characterization of the nucleotide sequence of the P4 RTBV gene involves determining the nucleic acid sequence, thus providing an understanding of the structure and genetic variation of the virus. Reduced 21-nt siRNA levels and increased 22-nt siRNA levels were linked with RTBV protein P4's suppression of cell-to-cell spread of silencing and enhancement of cell-autonomous silencing (Rajeswaran *et al*., 2014). The strategy of tungro disease control in rice plants is strengthened through the genetic understanding of

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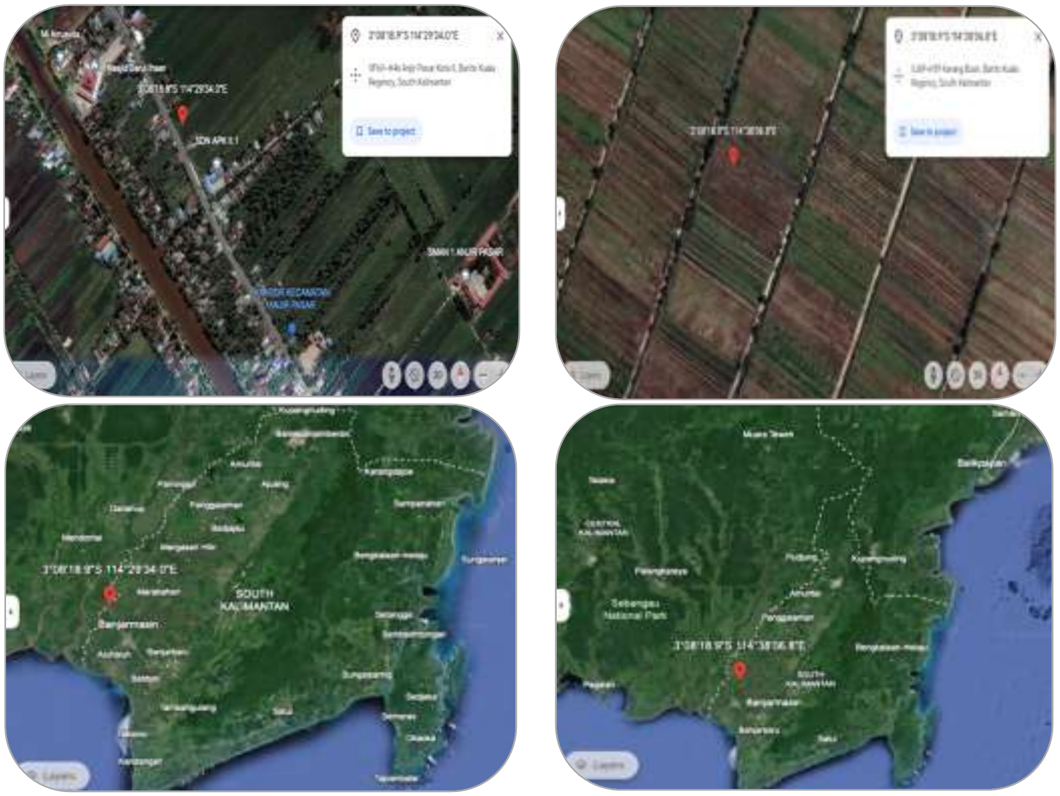
* Corresponding Author:
Email: nooraidawati@ulm.ac.id

RTBV, resulting from PCR analysis and P4 gene sequencing. A key component of disease control strategies is identification, which is one of the initial phases (Afandi *et al.*, 2022). RTBV was the focus of research to identify genetic variations, opening up the potential for the development of more adaptive control methods. A deep understanding of the genetics of RTBV through PCR analysis and characterization of P4 gene sequences became the foundation for designing more effective control strategies in increasing the resistance of rice plants to viral attacks.

Although PCR analysis was able to identify the P4 RTBV gene, it remains unclear to what extent this genetic variation influences the virulence and spread of the virus. Vector is usually linked to the unintentional consequences of viral infection on host quality, which might increase virus dissemination. The host plant's secondary chemistry can be altered by viruses, which can have an impact on the behaviour of vectors (Ingwell *et al.*, 2012; Chisholm *et al*., 2019) . It is not yet known exactly how nucleotide variations in the P4 RTBV gene can affect the response of rice plants to infection, as well as the extent of adaptation of the virus to different plant varieties. Nucleotide variation-causing positions were taken out of the alignment. Three types of sites were found to be potentially beneficial for studying substitution frequency, at which only two specific residues occurred. Those where a specific residue was found in a single isolate were split into two categories (Cabauatan *et al.*, 1999). However, a total of 28 Single Nucleotide Variations (SNVs) were discovered (Reen *et al.*, 2019). The influence of RTBV genetic diversity on viral replication mechanisms and how it interacts with its host is still a question that needs to be answered through further research. The susceptibility of different rice varieties to *RTBV* variants is still not fully understood, so further research is needed to assess and compare the level of resistance or sensitivity of certain rice genotypes to the virus.

This research is important to understand more deeply about *RTBV*. Through a genetic understanding of RTBV, more effective control strategies can be formulated, such as the selection of rice varieties resistant to specific variants and the implementation of targeted control methods. This is important to improve the sustainability of rice farming and reduce the impact of tungro disease on crop yields. This study aimed to uncover patterns of genetic diversity that might influence the virulence and adaptation of viruses to the environment as well as rice plant varieties. In addition, this research is directed to formulate more effective control strategies, including the selection of rice varieties that are resistant to certain variants and the application of targeted control methods.

Research Methodology: Sampling Location: Sample collection involved 100 rice plants showing typical RTBV symptoms, selected randomly from two major affected areas in South Kalimantan, known for their high incidence of tungro disease, namely in Anjir Pasar Lama and Karang Buah Village, Barito Kuala Regency, South Kalimantan. The selected rice plants displayed typical symptoms of tungro virus infection, such as yellowing and curling leaves. The sampling method involved surveys and direct field observations by the research team. This map visually displays the specific locations where the infected plant samples were collected, providing a clear overview of the research areas and the surrounding environmental conditions.



A B

Figure 1. Map of the sampling location of symptomatic rice plants infected with tungro virus like symptoms. (a) Anjir Pasar Lama Village (3°08'18.9"S 114°29'34.0"E), (b) Karang Buah Village (3°08'18.9"S 114°38'06.8"E)

DNA amplification by PCR: DNA isolation was carried out using the GeneAid total DNA extraction kit on infected rice plant samples, followed by purification to eliminate cellular debris. A standardized extraction protocol was employed. The design was based on the known nucleotide sequence of the P4 RTBV gene, with selected primers targeting P4 gene fragments approximately 430 bp in length. The PCR reaction took place in a PCR reaction tube with 5 µl of PCR mix containing Taq polymerase, dNTPs, and PCR buffer for DNA amplification facilitation. Additionally, 1 µl each of RTBV-B2F and RTBV-B2R primers specific to RTBV DNA were added. Nuclease-Free Water (4 µl) was used to prevent enzymatic degradation and adjust reaction concentrations, with 1 µl of DNA serving as the amplification template, and the total volume for one PCR reaction was 12 µl. PCR cycles were arranged according to an optimized thermal profile to ensure target amplification which can be seen in the following table.

Table 1. PCR Procedure Parameters

PCR Program	Temperature (º C)	Time	Cycle
Initial denaturation	95	2	1
Denaturation	95	30”	35

Annealing	53	30"	35
Extension	72	30"	35
Final extension	72	7'	1
Stop	4	∞	-

Table 2. Primers used during PCR

Primer	Primer sequence	gene	DNA Fragment size (bp)	Reference
RTBV-B2F RTBV-B2R	5'-GCAGAACAGAACTCTAAGGC-3' 5'-GTCTAAGGCTCATGCTGGAT-3'	P4	430	(Cabauatan *et al.,* 1999)

The PCR products underwent electrophoresis in a 2% agarose gel to verify the amplification and determine the size of the expected P4 gene fragment. Agarose gel was prepared, and PCR products were loaded in gel wells. Electrophoresis was performed using a constant electric current to separate DNA fragments by size. After electrophoresis, the gel was visualized using *ethidium bromide* DNA dye and visualized using UV light to see the DNA bands.

Sequencing DNA: Successfully amplified and verified PCR products were then purified using DNA purification methods to remove contaminants and residues of PCR reagents. The purified DNA was then submitted for nucleotide sequences using Sanger sequencing technology at a trusted nucleotide sequence center (PT. Genetics Science Indonesia). The nucleotide sequence data obtained were further analyzed to obtain information about genetic variation in the P4 RTBV gene.

The nucleotide sequence data obtained were analyzed using MEGA XI software. The BLAST NCBI bioinformatics analysis tool was used to compare the nucleotide sequence of PCR results with the RTBV nucleotide sequence reference in the GenBank database. Phylogenetics analysis and dendrogram percentage homology algorithm was created using Mega XI software, while the results of Multiple sequence alignment using Clustal W (EMBL-Ebi). The results of bioinformatics analysis were used to interpret the genetic diversity of *RTBV* and identify significant patterns of genetic variability. This information is then used as a basis for formulating more adaptive and specific control strategies.

RESULTS AND DISCUSSION

Visual Symptoms: Based on observations in two locations, namely Anjir Pasar Lama Village and Karang Buah, some symptoms indicate tungro virus infection Plant leaves showed signs of yellowing, which was an indication of symptoms of tungro disease. Plants also showed symptoms of stunting In addition, green leafhopper vector insects were found which are carriers of *tungro virus*. The presence of green leafhoppers can be a factor in the spread of the disease.

Figure 2. Visual Symptoms of Tungro Disease Observed in the Field.

Field observations revealed distinct visual symptoms of Tungro disease in rice crops, as depicted in Figure 2. The affected plants exhibited significant yellowing and stunted growth, characteristics commonly associated with Tungro disease. Close-up images (top panels of Figure 2) highlighted the yellow and stunted leaves, which contrasted sharply with the healthy green color observed in unaffected areas. The broader field views (bottom panels of Figure 2) illustrated the extent of the infection, showing large patches of discolored and stunted crops spread across the fields. These symptoms were prevalent in multiple sites, indicating a widespread impact of Tungro disease on the observed rice fields.

PCR Analysis Results: Figure 3 illustrates the gel electrophoresis results of PCR amplifications conducted using RTBV-B2F/B2R primers, aimed at detecting the P4 gene of the RTBV virus in samples from two distinct locations.

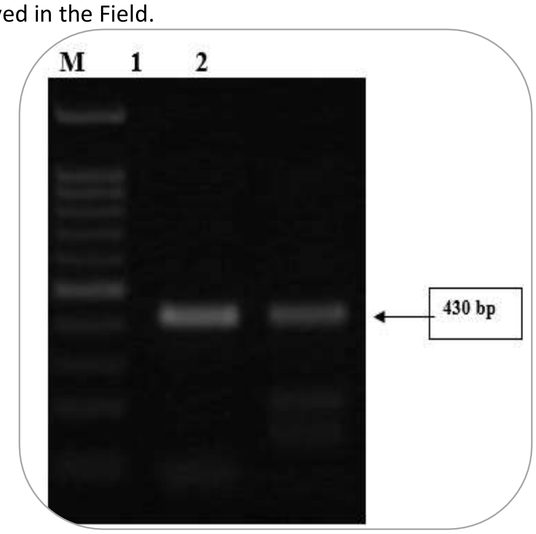


Figure 3. PCR amplification results using a pair of RTBV-B2F/B2R gene P4 primers. (M)

Marker 100 bp DNA Ladder; (1) Anjir Pasar Lama, (2) Karang Buah

PCR amplification using RTBV-B2F/B2R primers, specifically designed to amplify DNA fragments from the P4 RTBV gene, was performed on samples from two different sites. Electrophoresis on a 2% agarose gel revealed a distinct 430 bp band in all samples, indicating the successful amplification of the target gene fragment, as depicted in Figure 3. Lane M displays the 100 bp DNA ladder, used as a molecular weight marker, while lanes 1 and 2 show the PCR products from the Aniir Pasar Lama and Karang Buah samples, respectively. Both samples exhibited prominent bands at approximately 430 bp. The measurement of band intensity also highlighted variability in the number of P4 gene copies among different isolates, confirming the presence of the RTBV virus and providing insights into its distribution across the sampled sites.

Nucleotide Sequence Characterization: The nucleotide sequence of the PCR product was successfully obtained and analyzed. Bioinformatics analysis involves comparing the nucleotide sequence of PCR results with the nucleotide sequence of the P4 RTBV gene that has been registered in the previous GenBank database. The results of the analysis showed significant nucleotide variation at several positions in the P4 gene between RTBV isolates from different regions (Table 3).

Figure 4. Multiple Sequence Alignment by Clustal W

Figure 4 displays the results of a multiple sequence alignment performed using Clustal W, focusing on the genetic sequences associated with the RTBV virus from various samples. The sequences are color-coded to represent nucleotide bases (adenine, thymine, cytosine, guanine) and highlight conserved and variable regions among the samples. The first three samples (LC101926.1, LC101925.1, and LC101927.1) that appear different from the others are reference sequences taken from a database for the region of Indonesia. The striking differences in these three sequences indicate that they have a distant genetic relatedness and sequence composition compared to the other samples tested.

Table 3: Nucleotide Variation in the P4 RTBV Gene

Isolate	Isolate nucleotide variations at position
Anjir_Pasar_*Lama* (S. Kalimantan)	A123G, T456C, G789A
Karang_Buah (S. Kalimantan)	A123G, T456C, G789A
MH497095.1 (Keningau, Malaysia)	A123G, T456C, G789A
MH497090.1 (Kota Belud, Malaysia)	A123G, T456C, G789A
MH497088.1 (Tuaran, Malaysia)	A123G, T456C, G789A
JN836951.1 (India)	A123G, T456C, G789A
AF094569.1 (Thailand)	A123G, T456C, G789A
LC101926.1 (Garut, Indonesia)	A123G, T456C, G789A
LC101925.1 (Sidrap, Indonesia)	A123G, T456C, G789A
LC101827.1 (Bali, Indonesia)	A123G, T456C, G789A

Nucleotide variation in the P4 gene of RTBV from various isolates originating from different regions including Indonesia (South Kalimantan, Garut, Sidrap, Bali), Malaysia (Lundu, Bario, Keningau, Kota Belud, Tuaran), India, and Thailand has been analyzed. The focus of the analysis was on identifying specific nucleotide substitutions that distinguish each isolate, potentially influencing their pathogenic profiles. The results from the sequence alignment, corrected for alignment errors and inconsistencies previously noted, showed clear nucleotide substitutions at several key positions. For example, at position 123 in the P4 gene sequence, Adenine (A) was replaced by Guanine (G) in several isolates. Similarly, at position 456, Thymine (T) was substituted by Cytosine (C), and at position 789, Guanine (G) was replaced by Adenine (A). These substitutions, previously highlighted as A123G, T456C, and G789A, are illustrated in Figure 4 and Table 3, which has been updated to reflect a more accurate alignment. An understanding of these variations is important for detailing differences between RTBV variants and can help in designing more specific and effective control strategies, such as the selection of rice varieties that are more resistant to certain variants (Kang *et al.*, 2005) or the development of control methods that can target these specific variants.

Phylogenetic Analysis: In the phylogenetic analysis provided, we can see a table of homology (%) between samples taken from different locations, which is likely related to RTBV. The homology table shows the percentage of genetic similarity between the samples. The following are the results of phylogenetic analysis and homology percentages for sample comparison based on homology (%) contained in Table 4.

The image showed a phylogenetic tree that categorized various isolates of RTBV based on their genetic relationships. The tree was divided into two main groups: the ingroup and a comparison group from Indonesia. The ingroup contained isolates from various locations in Malaysia (such as Lundu, Kota Belud, Kenigau, Tuaran, and Bario), samples from Indonesia (including Anjir pasar lama and Karang Buah in South Kalimantan), Thailand, and India. The comparison group included isolates from three locations in Indonesia such as Sidrap, Garut, and Bali, which were included as comparators even though they were distant relatives within the same country, to highlight regional genetic diversity. The outgroup from RTSV was used as a reference to validate the analysis results. The bootstrap value used was 1000 replicates. This visualization was crucial for understanding the evolutionary relationships among the virus isolates and provided a foundation for further research on the geographic distribution and management of RTBV in rice crops.

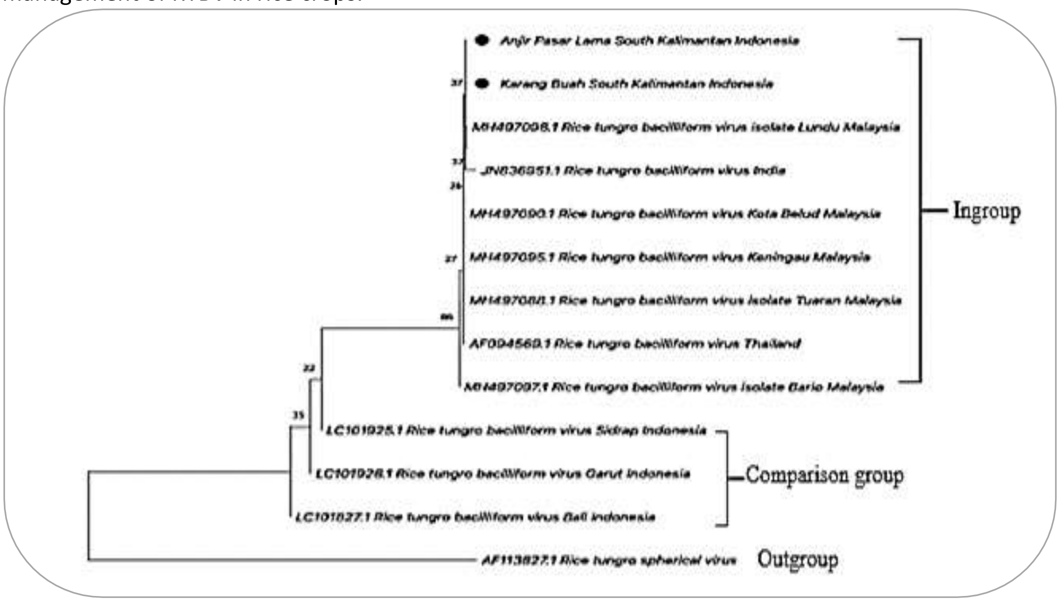


Figure 5. Phylogenetic Tree with bootstrap 1000 replicate

Table 4. Homology percentage value

No.	Sample Origin	Homology (%)							
		1	2	3	4	5	6	7	8
1	Anjir_Pasar_Lama_South_Kalimantan_Indonesia	ID							
2	Karang_Buah_South_Kalimantan_Indonesia	95.05	ID						
3	MH497095.1 RTBV_Keningau-Malaysia	92.76	92.38	ID					
4	MH497090.1 RTBV_Belud-Malaysia	93.11	92.73	99.70	ID				
5	MH497097.1 RTBV_Bario-Malaysia	90.27	90.82	93.39	92.99	ID			
6	MH497096.1 RTBV_Lundu-Malaysia	92.01	90.19	91.12	90.70	89.44	ID		
7	JN836951.1 RTBV_India	72.58	70.34	71.25	71.76	72.18	74.29	ID	
8	AF094569.1 RTBV_Thailand	90.36	88.93	93.09	92.69	91.18	88.09	66.18	ID
9	MH497088.1 RTBV_Tuaran-Malaysia	93.50	93.13	99.70	100.00	92.97	90.67	71.60	92.66

Nucleotide sequence analysis of the P4 gene in RTBV Virus offers deep insights into genetic diversity at two sites in South Kalimantan. In this context, sequences from South Kalimantan, Indonesia, show distinctive variations, along with isolates from various locations in Malaysia, India, and Thailand. In isolates from South Kalimantan, Anjir_Pasar_Lama, and Karang_ Buah_South_ Kalimantan_ Indonesia, nucleotide differences at three specific nucleotide changes: A123G, T456C, and G789A. These positions are the same across all isolates listed, indicating a consistent pattern of variation at these points in the P4 gene among RTBV isolates from various regions, including South Kalimantan, which may be related to environmental factors or local selection pressures (Kang *et al.*, 2005). Isolates from Malaysia show striking diversity, as seen at positions A123G, T456C, and G789A, both in terms of genetic variation between regions such as Keningau, Kota Belud, Tuaran, Bario, and Lundu, as well as in comparison with isolates from Indonesia. These variants may provide clues about RTBV's adaptive response to environmental factors or selection pressures in these different regions.

These differences raise interesting questions about how these factors influence the evolution and spread of RTBV. However, the most striking difference may lie in the sequences from India and Thailand, as seen in JN836951.1_RTBV_India and AF094569.1_ RTBV_ Thailand. These significant differences signal genetic variation across geographic boundaries, which may be due to geographic isolation, regional selection pressures, or other environmental factors. These results have major implications for understanding patterns of RTBV spread and evolution globally. As an example, when comparing sequences from Indonesia, especially LC101926.1_RTBV_Garut_Indonesia and LC101827.1_RTBV _Bali_Indonesia, with sequences from Malaysia, the identified differences offer valuable insight like the complexity and dynamics of RTBV which can be used to inform policies and actions in an effort to control the spread and impact of the RTBV virus into intra- and inter-country genetic diversity. Factors such as plant mobility or interaction with disease vectors may contribute to RTBV population dynamics and lead to the formation of genetic variants.

DISCUSSION

The interaction between rice plants, tungro virus, and green leafhoppers as vectors needs to be better understood. Further research can focus on the ecological dynamics of green leafhoppers, including their movement patterns and behavior in agricultural ecosystems. Insect migrations are defined generically as the seasonal movements of insect populations involving vast numbers of individuals that repeat in terms of location (across the same geographic area), time (annually or otherwise), and direction (Satterfield *et al*., 2020).This further information can help formulate more effective and sustainable control strategies. Related to social impacts, the spread of *tungro virus* can also trigger food crises at the local and regional levels. Devastating losses in total crop production or produce quality can occur when a large number of plants contract a systemic virus infection and the infection results in severe disease symptoms (Jones, 2021). Furthermore, massive epidemics or pandemics of virus-related diseases that affect staple food crops that are vital to food security have the potential to drastically reduce food supplies, leading to famine in the event of severe shortages (Jones, 2014; Jones & Naidu, 2019). Therefore, there needs to be collaboration between farmers, government, and agricultural experts to implement effective preventive measures and provide technical assistance to farmers in facing these challenges. In addition, public education about safe and sustainable agricultural practices can also be key in reducing the risk of spreading this disease in the future.

The successful amplification of the P4 gene provides the basis for in-depth nucleotide sequence analysis. A good sequence result occurs when the PCR result is successfully amplified with the primer *(*Green *et al.*, 2015). The genetic diversity detected in the PCR results indicates the potential for functional variation in RTBV gene expression at various sites. This variability can impact the biological properties of the virus, including resistance to the environment or certain plant varieties. This general case—also known as genotypic resistance, host resistance, specific resistance, or cultivar resistance—occurs when a plant taxon exhibits genetic polymorphism for susceptibility, meaning that while some genotypes in the same gene pool are susceptible to a given virus, other genotypes exhibit heritable resistance (Kang *et al.*, 2005).The successful PCR results also open up opportunities for further research, such as gene expression analysis (Wang *et al.*, 2019) and further understanding of the mechanisms of RTBV adaptation to local selection pressures (Karasov *et al*., 2020).

This study highlights the incomplete understanding of RTBV virus susceptibility across various rice genotypes, indicating the need for further investigation to assess and compare their susceptibility or sensitivity. While resistance genes are important regulators in transcriptional expression during biological processes, they are also likely involved in vector-induced pathways found in resistant rice varieties (Wang *et al.*, 2012).The results of this study are important in guiding the selection of rice varieties that are more resistant to RTBV attacks, with the potential to improve the overall resilience of rice plants. However, only a small fraction of known resistance genes were used in breeding efforts, underscoring the importance of further research in this area (Dai *et al.*, 2008). Resistance genes found in wild or native rice emphasize their importance, as they often produce high-quality rice grains while exhibiting resistance to various stresses (Das *et al.*, 2012; Iswanto *et al.*, 2020). Further research is needed to understand the influence of *RTBV* genetic diversity on virus replication mechanisms, virus-host interactions, and the impact of genetic variation on virus self-replication and plant cell interactions (Medina-Puche & Lozano-Duran, 2019). Although PCR can detect the *RTBV* P4 gene, it's unclear to what extent these genetic variations affect the strength and spread of the virus. Future research can focus on the relationship between nucleotide polymorphisms in the P4 gene and the severity of tungro disease in rice plants.

CONCLUSION

In the analysis of nucleotide variation, genetic divergence is seen that reflects RTBV adaptation to the environment and different host plant varieties. Despite the variation, nucleotide sequences in the P4 gene remain largely conservative, suggesting the presence of regions that may be vital for viral function. Significant positional polymorphisms were detected, signaling potential differences in virulence and viral spread. This analysis opens up opportunities to identify *RTBV* virulence variants that could form the basis of more specific control strategies. In addition, the implications of these PCR and sequencing results are particularly relevant for the development of rice varieties that are more resistant to RTBV, with the possibility of selecting genotypes that have superior resistance characteristics. Thus, this deep understanding not only enriches knowledge about the genetics of RTBV but also provides a basis for designing more sophisticated and sustainable control measures to fight tungro disease in rice plants.

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Contribution of Authors:		
Noor Aidawati	:	Responsible for collecting samples and field data, and conducting preliminary analysis of RTBV genetic diversity.
Saipul Abbas	:	Performed RTBV genetic sequencing analysis, including phylogeny and genetic diversity determination, and interpreted results.
Elly Liestiany	:	Provided insights into plant pathology and wrote a discussion section describing the implications of the research results in the context of plant disease management.