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Disease incidence and molecular diversity of Tungro Virus on Rice (*Oryza sativa*) in Bengkulu, Indonesia

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Abstract There has been no updating data on the incidence of viral diseases that attack rice plants in Bengkulu. Updating data on viral disease distribution status is an initial step to prevent disease outbreaks. The disease incidence and molecular characterization of the tungro virus in Bengkulu, Indonesia wasc reported. Detection of viruses from field samples was amplified Rice Tungro Spherical Virus (RTSV) and could not be amplified RTSV from other field samples. Specific DNA fragments of ±787 bp were successfully amplified using specific primers for the coat protein gene of RTSV. Spesific DNA fragments of 1400 bp were succesfully amplified from Rejang Lebong, Bengkulu Tengah, Bengkulu Utara, and Bengkulu city using Rice Tungro Bacciliform Virus (RTBV) spesifics primers DAF and DAR. The results showed that the RTBV rice isolates from Bengkulu Utara and Rejang Lebong were closely related to the RTBV Seberang Perai isolates from Malaysia (MK552377).

Keywords: Tungro, RTSV, RTBV, RT-PCR

Introduction

Rice is the most widely consumed staple food in Asian countries including Indonesia. The viral disease is an essential constraint for rice production in Indonesia. Yield loss due to tungro diseases are commonly reported during the growing season. Rice tungro disease (tungro) is one of the most destructive rice diseases in South and Southeast Asia, where the disease's epidemics have occurred since the mid-1960s (Azzam *et al.,* 2000). In the

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Philippine dialect *Ilocano*, tungro means degenerated growth (Chancellor *et al.,* 2006). Tungro is also called by various names in different countries; Indonesia: *mentek or penyakit habang*; Malaysia: *penyakit merah*; Thailand: yellow-orange leaf; and India: leaf yellowing.

Tungro is caused by two viruses (Hibino, 1996): an RNA virus, the rice tungro spherical virus (RTSV), and a DNA virus, the rice tungro bacilliform virus (RTBV). *Rice tungro bacilliform virus* is a pararetrovirus and is a member of the family *Caulimoviridae*. RTBV depends on the helper produced by RTSV for its transmission. It is the RTBV that is mainly responsible for the severe tungro symptoms. RTBV has an 8-kbp double-stranded DNA genome with two discontinuities, one in each strand (Hull, 1996). Rice plants infected by RTBV and RTSV show typical tungro symptoms: stunting and yellow or orange-yellow discolouration of the leaves (Hibino, 1996). Infected plants also have a reduced number of tillers and may show rust-coloured spots on the leaves. The degree of stunting and leaf discolouration varies with rice varieties, strains of the viruses, the plant's age when infected, and the environment.

Some of the critical factors that influence the development of tungro disease are the population of vector insects, *Nephotettix virescens* Distant, which is relatively high, the availability of inoculum sources, the presence of asynchronous cropping patterns and the presence of susceptible varieties. The first time tungro attacked 25,000 ha of rice fields in Indonesia in 1983-1984 (Azzam *et al.,* 2000). In the last ten years, tungro disease has spread in 22 provinces, including South Sulawesi, NTB, West Java (2,443 ha), East Java (1,150 ha), Bali (1,093 ha), Central Java (710 ha), and Bengkulu (521 ha).

There has been no data on the viral disease incidence that attack rice plants in Bengkulu. Identification of the important virus on rice needs to be done. Rice tango tungro has experienced an outbreak and increaseed in the green planthopper population in various regions in Indonesia. Updating data on rice virus types and their distribution status is an initial step to preventing disease outbreaks. The objective was to study the disease incidence of tungro virus and the molecular diversity of the tungro virus in Bengkulu, Indonesia.

Materials and methods

Survey and collection of field samples

Rice leaves showing typical virus infection symptoms (stunting and yellow or orange-yellow discolouration of the leaves) were collected from several paddy fields (17 areas, five districts: Rejang Lebong, Bengkulu Utara, Bengkulu Tengah, Bengkulu Selatan, and Seluma) in Bengkulu province.

Young leaf samples were collected from the plant's age 4-8 weeks after planting. Samples were taken with purposive-random sampling according to the virus infection symptom. The observation was conducted directly in the field with a 500-1000 m^2 area. Leaf samples were placed in plastics bags and carried to the laboratory for virus detection. At the same time, we determined disease incidence by counting the number of plants that show the symptoms from 30% of the plant population.

RTSV detection by the reverse transcription-polymerase chain reaction

Virus detection was done at the Laboratory of Biotechnology and Genetics at Department of Biology, Faculty of Mathematics and Natural Sciences, University of Bengkulu. Detection of RNA viruses by the Reverse transcription-polymerase chain reaction (RT-PCR) method using a pair of primers specific to RTSV. According to the RNA virus's manufacture method, we extracted total RNA from symptomatic leaves using a Promega RNA extraction kit. DNA amplification was carried out using a specific primer for RTSV-F2 (GAA GAA GCC TAT CAT GYT CGCGT) dan RTSV-R2 (CCT CCA CGA TAT TGT ACG AGG) for RTSV. The reverse transcription reaction was carried out with one-step RT-PCR with a total volume of 25 µl consisting of 2 µl of total RNA, F and R primers each of 2.5 µl, 0.05 µl of Revest aid NZY, 2.5 µl of DTT 50 mM, 0.2 µl Rnase inhibitor, 12.5 µl GoTaq Green, and 2.75 µl ddH2O. The reverse transcription reaction was carried out at a temperature of 42 ℃ for 60 minutes, predenaturation of 94 ℃ for 5 minutes for one cycle, then 40 cycles including denaturation of 94 ℃ for 1 minute, annealing 50 ℃ for 1 minute, synthesis of 72 ℃ for 2 minutes and then the extension of 72 ℃ for 10 minutes and finally cool down to 4 ℃ until the PCR program is finished. Then, visualise cDNA in electrophoresis by running at a voltage of 50 volts for 50 minutes. Agarose gel is soaked in EtBr for 15 minutes. After that, washed ddH2O then visualised the agarose gel on the documentation gel (Axygen).

RTBV detection by the polymerase chain reaction

For the detection of DNA virus, we extracted total DNA from symptomatic leaves according to the method of (Doyle JJ and Doyle JL 1990) with minor modification. Leaf tissue was ground in sterile mortar in liquid nitrogen and then 500 µl extraction buffer added to the leaf powder. The extraction buffer contained 100 mM Tris pH 8.0, 1.4 mM NaCl, 20 mM EDTA pH 8 and 0. 2% (v/v) β-mercaptoethanol. The extraction buffer was autoclaved,

and 2% (v/v) polyvinyl pyrrolidone (PVP) and 2% CTAB were added immediately before use. After grinding, 500 µl aliquots were transferred to a 2 ml microtube and incubate for 30 minutes at 65 ℃ with mixing to avoid the homogenate's aggregation. The extract added 500 µl of chloroform: isoamyl alcohol (24:1), and the mixture was vortexed thoroughly. Then the microtube centrifuged for 15 minutes at 13 000 rpm. The supernatant was then transferred to a new tube, and protein precipitated by adding isopropanol 2/3 x volume of supernatant and centrifuged for 10 minutes at 12 000 rpm. The pellet was washed with ethanol 70% (v/v) and centrifuged 5 minutes at 8 000 rpm. The pellet was dried and resuspended by 100 µl nuclease-free water. This total DNA extract was stored at -20 ℃ for further use. The RTBV genome was amplified by the PCR technique using an RTBV DAF (5'- GGA TTC CGG CCC TCA AAA ACC TAG AAG -3') and primer DAR RTBV (5'- GGG GGT ACC CCC CTC CGA TTT CCC ATG TATG -3'). PCR reactions were prepared in 25 µl total volume, containing go taq green master mix (2x), ten mM each primer, 1 µl DNA template, and ddH2O. The amplification was performed with 5 minutes at 94 ℃ for pre-heating, followed by 34 cycles of denaturation (1 minute at 94 ℃), annealing (1 minute at 62.2 ℃), and extension (2 minutes at 72 ℃). The last cycles were followed by 72 ℃ for 10 minutes and decreased to 4 ℃. Agarose gel electrophoresis was used to visualise the PCR product.

Sequencing and molecular diversity

The PCR products of RTBV from Bengkulu Utara (K44 BU, K22 BU, K21 BU) and Rejang Lebong (R22 RL) were sequenced by FirstBase Sequencing Int. (Selangor, Malaysia). BLAST was performed to find similarity of sequenced DNA with reference data base in GeneBank NCBI. We compared the homology sequences of viruses to isolates from other countries in the GenBank database as implemented in Bio Edit v7.05. Phylogenetic analysis was constructed by MEGA v6.0 software with the neighbor-joining algorithm and bootstrap value 1000 repetitions (Tamura *et al*., 2013).

Results

Disease incidence

The results of disease incidence are summarised in Table 1. Our research survey was conducted in 14 paddy field areas covering six districts in Bengkulu province (Rejang Lebong, Seluma, Bengkulu Utara, Bengkulu Tengah, Bengkulu Selatan, dan Bengkulu city) from April to September 2019

Symptoms due to virus infection and those caused by abiotic stress (insecticide, herbicide, nutritional deficiency, and drought stress) were observed in several fields. The most common symptom found in the field was stunting and yellow or orange-yellow discolouration of the leaves at rice plant's ages 4-8 weeks after planting.

Tungro disease symptoms were found in all the surveyed regions, with tungro disease incidence ranging from 0.7 to 23% (Table 1). Tungro disease in rice plants is associated with vector insects, plant variety, and weeds as virus hosts. Based on field observations, 1 to 8 heads/100m^2 (data are not shown). The green leafhoppers population is shown as the vector insect control threshold.

Table 1. Survey location, rice plant, and disease incidence (per cent) of tungro disease in Bengkulu province, Indonesia

No.	Location		Variety	Age of plant (week after planting)	Diseas Incidence (%)
	District	Village, sub-district			
1	Rejang Lebong	Batu Panco, Curup Utara	Ciherang	6	2.13
2	Rejang Lebong	Rimbo Recap, Curup Selatan	IR Lampung	4	1.42
3	Rejang Lebong	Rimbo Recap, Curup Selatan	unknown	6	3.64
4	Bengkulu Tengah	Pondok Kelapa	Inpago	6	32.5
5	Bengkulu Utara	Sumber Sari, Kemumu	unknown	6	8.57
6	Bengkulu Utara	Sumber Sari, Kemumu	unknown	8	23
7	Bengkulu Utara	Pematang Balam, Hulu Palik	Batu Bara	4	0.7
8	Bengkulu Utara	Batu Roto, Hulu Palik	unknown	4	0.42
9	Seluma	Air Latak, Seluma Barat	Ciherang	4	0.5
10	Seluma	Taba, Talo Kecil	Ciherang	6	2.73
11	Seluma	Taba, Talo Kecil	Cigeulis	4	1.79
12	Bengkulu Selatan	Pagar Bunga, Kedurang	Cigeulis	4	2.05
13	Bengkulu Selatan	Rantau Sialang, Kedurang	Mekongga	4	4.31
14	Bengkulu Selatan	Darat Sawah Ulu, Seginim	unknown	4	1.42
15	Bengkulu city	Semarang, Bengkulu city	Aromatik	7	0.89
16	Bengkulu city	Semarang, Bengkulu city	Cigeulis	7	2.54
17	Bengkulu city	Semarang, Bengkulu city	Sentani	7	1.24

Detection of RTSV by Reverse transcription-polymerase chain Reaction

Detection of viruses from field samples was able to amplify RTSV from Bengkulu city but could not amplify RTSV from other field samples. Specific DNA fragments of ±787 bp (figure not shown) were successfully amplified from Bengkulu city using specific primers for the coat protein gene of RTSV. The DNA bands obtained were not sufficient to proceed to the sequencing.

Detection of RTBV by polymerase chain reaction

Spesific RTBV DNA fragments of 1400 bp were succesfully amplified from Rejang Lebong, Bengkulu Tengah, Bengkulu Utara, and Bengkulu city using RTBV spesifics primers DAF and DAR (figure 1). RTBV was detected in four districts in infected samples showing stunting and orange-yellow discolouration on leaves. However, the RTBV fragment was not successfully amplified from samples from Bengkulu Selatan and Seluma (Figure 1). The cDNA concentration of samples from Bengkulu Tengah and Bengkulu City were too low for sequencing. Three positive samples from Bengkulu Utara (K44-BU, K22-BU, K21-BU) and one sample from Rejang Lebong (RR-22) then chosen to represent two locations for sequencing.

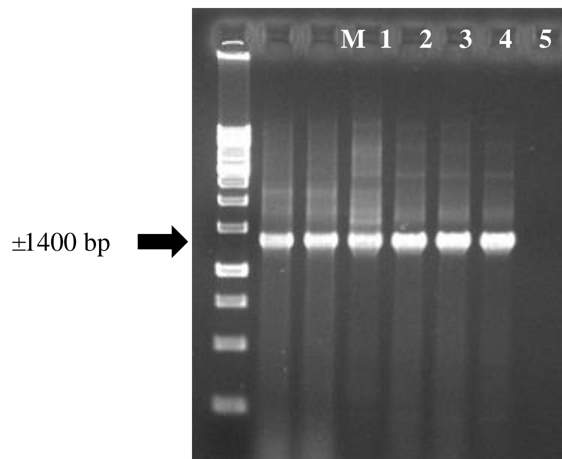

Figure 1. Electrophoresis analysis of PCR for RTBV in symptomatic rice leaves using 1% agarose gel stained with ethidium bromide. PCR products: (M) marker 1 kb DNA ladder, (1-2) RR-22 from Rejang Lebong; (3-5) DNA RTBV from Bengkulu Utara; (6) positive control; (K-) negative control from healthy rice plant

Molecular diversity

Analysis of nucleotide sequence

DNA from Rejang Lebong district and Bengkulu Utara district were partially sequenced. Nucleotide sequences after BLAST with NCBI Genebank database showed that RTBV from Indonesia were genetically close with RTBV nucleotide accesion number AF076470.1 and MK552377.1 from Malaysia. Almost all samples from Bengkulu, Indonesia had similarity 93-95% with RTBV isolate from Malaysia (Table 2).

Table 2. Similarity percentage of nucleotide sequences of the partial segment of RTBV from test sample comparison to RTBV isolates from NCBI Genebank database

Accession	Area/Country	K44_BU	K21_BU	K22_BU	R22_RL
K44_BU	North Bengkulu/Indonesia		100	100	99
K21_BU	North Bengkulu/Indonesia	100		100	99
K22_BU	North Bengkulu/Indonesia	100	100		99
R22_RL	Rejang Lebong/Indonesia	99	99	99	
AF076470.1	Serdang/Malaysia	94	94	94	93
MK552377.1	Seberang Perai/Malaysia	95	95	95	94
AF094570.1	Thailand	94	94	94	93
LC101923.1	Lombok/Indonesia	93	93	93	92
LC101922.1	South Minahasa/Indonesia	93	93	93	92
LC101920.1	Padang/Indonesia	94	94	94	92
LC101828.1	Bogor/Indonesia	93	93	93	92
LC081238.1	Magelang/Indonesia	93	93	93	92
AF094573.1	Thailand	93	93	93	92
M65026.1	Philippines	93	93	93	92
LC101924.1	West Sulawesi/Indonesia	93	93	93	92
AF220561.1	Thailand	92	92	92	91
AF113831.1	Philippines	93	93	93	92
LC101827.1	Bali/Indonesia	93	93	93	92
D10774.1	Japan	92	92	92	91
AF113830.1	Philippines	92	92	92	91
AF113832.1	Philippines	92	92	92	91

Phylogeny of RTBV rice isolates from Bengkulu Utara and Rejang Lebong

Neighbor-Joining (NJ) method was used to construct phylogenetic trees using nucleotide sequences of RTBV from test samples and RTBV from GeneBank. The dendogram of molecular affinity of RTBV based on nucleotide sequence showed that the RTBV rice isolates from Bengkulu Utara and Rejang Lebong were separated from other group. The phylogenetic tree analysis of RTBV isolates based on nucleotide sequences and amino acids compared with the GenBank database's corresponding isolates showed that the RTBV isolates were divided into four clades (Figure 2). The results showed that the RTBV isolates from Bengkulu Utara (K44 BU, K22 BU, K21 BU) and Rejang Lebong (R22 RL) were closely related to the RTBV Seberang Perai isolates from Malaysia (MK552377). Our results support the previous report that there is a clear correlation between CP sequences variations and the virus isolates' geographic origins in Indonesia (Praptana *et al.,* 2017).

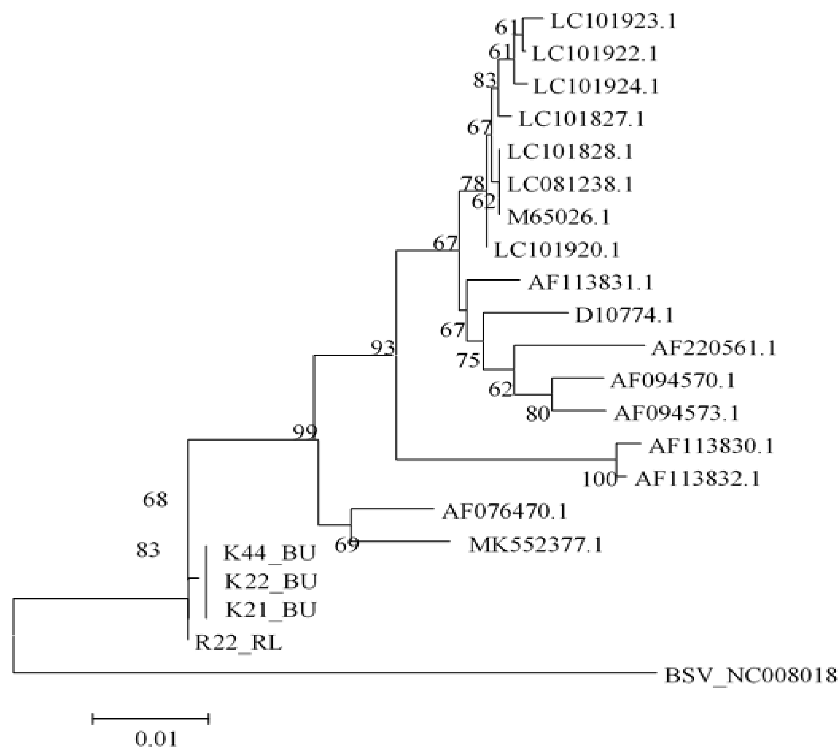

Figure 2. Phylogenetic tree based on the alignments of the common region sequences of RTBV from Bengkulu with other selected RTBV (GeneBank database)

Discussion

Many factors influence the incidence of tungro disease. Asynchronous planting can cause the tungro outbreak because it will let vectors continue feeding, transmit the virus, lay eggs, and multiply. They are using inappropriate varieties, applying a high number of fertilizers containing nitrogen and high temperature, which will accelerate the occurrence of tungro. Transmission of the tungro virus by leafhoppers occurs semi-persistently, it can be transmitted efficiently by its vectors in minutes to hours. A planthopper carrying the virus has the potential to spread the tungro virus inoculum for several hours. The leafhopper did not transmit RTBV from plants infected with RTBV alone, while RTSV was efficiently transmitted from plants infected with RTSV alone. RTBV transmission occurred when the leafhoppers were previously fed on RTSV-infected plants. Similar results were obtained when nymphs were used for transmission. Adult leafhoppers retained RTSV for three days after the termination of acquisition access. However, when the leafhoppers were given access to RTBV-infected plants, RTBV transmission occurred seven days after the termination of acquisition access. This indicates that RTSV itself may not be the "helper factor" for RTBV transmission. The "helper factor" for RTBV transmission was lost after molting (Hibino, 1996).

The variation of nucleotide bases in the ORF2 sequence occurred in six RTBV strains from Philippines, namely: Phi1, Phi-2, Phi-3, G1, G2, and Ic the Serdang strain from Malaysia (Cabauatan *et al.,* 1999). Analysis of intergenic region (IR) sequences and some ORF1 sequences in several RTBV isolates originating from Indonesia (Subang), Malaysia (Serdang), Philippines, and Vietnam showed several variations in nucleotide bases (Azzam *et al.,* 2000). Nucleotide base substitution also occurred in some ORF4 sequences in several RTBV isolates from the same region in Philippines (Villegas *et al.,* 1997). A significant difference between RTBV isolates from the Indian subcontinent and Southeast Asia was deletions of 64 bp in the ORF4 sequence of Southeast Asian isolates (Fan *et al.,* 1996). The substitution of some nucleotide bases on the end of the ribonuclease H terminal causes changes in two types of amino acids that differentiate between West Bengal and Andhra Paradhes' RTBV isolates (Nath *et al.,* 2002). The nucleotide based substitution in the ORF2 RTBV isolates of West Bengal, Andhra Paradhes, and Kanyakumari did not cause amino acid changes so that the amino acid sequence similarity of the three was 100% (Sharma and Dasgupta, 2012). The genetic diversity and virulence of the tungro virus in Southeast Asia are different from that of the tungro virus in South Asia (Azzam and Chancellor, 2002).

This group is separated from the Serdang strain and other groups consisting of isolates from Thailand, India and two strains from Philippines. The Serdang strain position that was separated from the other isolates and strains was in line with the results of the study by Cabauatan *et al.* (1999). The effects of grouping based on the partial ORF2 sequence differed from the relationship dendrogram of RTBV isolates based on a variety of intact DNA sequences, namely the West Bengal, Kanyakumari, Andhra Paradhes, and Chinsura isolates were grouped far apart from several isolates and strains from Thailand, Malaysia, and Philippines (Sharma *et al*., 2011).

Kinship analysis based on IR sequences and partial ORF1 showed that Indonesia's two RTBV isolates were separated from isolates from Malaysia, Philippines, and Vietnam (Azzam *et al.,* 2000). This indicates that the variation of nucleotide bases in certain parts of the entire RTBV DNA sequence have a different degree of similarity between other isolates and strains. The genetic relationship between RTBV and amino acids in part of the ORF2 sequence was different from the kinship-based on the nucleotide base sequence (Figure 2). Daerah Serdang (Malaysia) and Seberang Perai (Malaysia) isolates became separate groups from the other two groups, while Japan, Thailand, Indonesia (Bogor, Bali, Lombok, West Sulawesi, Magelang, Padang) isolates were still joined in one group with Philippine isolates. Changes in position also occurred in isolates from Thailand who were far apart from the Indian group and were members of the Serdang group. The positions of isolates and strains from Philippines and isolates from India were more consistent. The similarities of nucleotide bases and amino acids in the whole ORF2 sequence between Phil 1 and West Bengal isolates were 81.7% and 87.3%, respectively, so they are somewhat distant in genetic kinship (Nath *et al.,* 2002).

The genetic diversity and relationship between RTBV and RTSV isolates in various endemic areas are very important information to determine control strategies using resistant varieties, including through the assembly and improvement of specific resistant varieties of tungro virus isolates, rotation of resistant varieties, and planting of multivariates with different resistance genes (Tiwari *et al.,* 2012)(Skelsey *et al.,* 2004). Several isolate-specific tungro-resistant varieties have been produced from the assembly and repair of types using suitable resistant parental material to deal with specific tungro virus isolates from endemic areas (Muliadi, 2017). Tungro resistant varieties containing various resistance genes can be used in a variety rotation program to control tungro and maintain the durability of resistance to viruses and green leafhoppers. However, the durability of resistance can decrease because selection pressure can increase the virulence, so it is necessary to monitor the

virulence of the pathogens against each resistant variety in an endemic area on a temporal basis (Fabre *et al.,* 2012) (Janzac *et al.,* 2009).

Tungro disease caused by RTBV infection which found to attack rice plants in Rejang Lebong, Bengkulu Tengah, Bengkulu Utara, and Bengkulu city with a low incidence. The incidence of tungro in Bengkulu is still sporadic, and it should be noted because it is related to its vector insect population dynamics of green leafhoppers. RTBV isolates from Bengkulu Utara (K44 BU, K22 BU, K21 BU) and Rejang Lebong (R22 RL) were genetically closed with the RTBV Seberang Perai isolates from Malaysia (MK552377).

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