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SHORT COMMUNICATION

Molecular characterization of *Rice ragged stunt virus* and *Rice grassy stunt virus* on Rice in Gianyar, Bali, Indonesia

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ABSTRACT

Rice stunt disease is one of the causes of rice harvest failure. It is caused by the infection of *Rice ragged stunt virus* (RRSV) and *Rice grassy stunt virus* (RGSV) infection. Information about disease severity and the molecular characteristics of stunt viruses in Indonesia is still limited. Thus, this research aimed to determine the disease severity and the genetic diversity of rice stunt viruses in Gianyar, Bali. The research method consisted of observation of incidence and disease severity in the field and virus detection by reverse transcription-polymerase chain reaction (RT-PCR) using primers specific for RRSV and RGSV. The observation of the disease incidence and severity were performed in seven districts in Gianyar Regency, Bali, namely Blahbatuh, Gianyar, Payangan, Sukawati, Tampaksiring, Tegallalang, and Ubud. Stunt disease was found in all observation sites. High stunt disease incidence (> 44%) was found in three districts: Ubud, Tampaksiring, and Payangan, while the low disease incidence rate of <10% was found in Blahbatuh and Gianyar Districts. The highest stunt disease severity occurred in Tampaksiring District (60.82%), while the lowest severity occurred in Gianyar District (18.84%). The IR-64 and Ciherang cultivars are vulnerable to rice stunt disease infection. The highest homology of RRSV and RRGV nucleotides was found with Vietnam isolates being >98% and >97%, respectively. The phylogenetic analysis showed that Indonesian isolates of RRSV and RGSV were clustered in the same group as Vietnam isolates.

Key words: disease incidence, disease severity, genetic diversity, RGSC, RRSV

INTRODUCTION

The stunt virus is the primary agent causing rice crop failures in several countries. Stunt diseases consist of rice ragged stunt disease caused by the *Rice ragged stunt virus* (RRSV) and rice grassy stunt disease caused by the *Rice grassy stunt virus* (RGSV) (Helina et al., 2018). Stunt virus is persistently transmitted by its vector, the brown planthopper. The brown planthopper can transmit RGSV until the end of its lifespan. However, it cannot transmit the virus to its offspring through eggs (Huang et al., 2015). Other than the insect vector, several weeds around the rice crops also act as alternative hosts for RGSV. Weed species naturally infected by RGSV are *Axonopus compressus, Eleusine indica, Echinochloa colona,* and *Monochoria vaginalis* (Listihani et al., 2023). On the other hand, RRSV can be found on several weeds growing around rice crops, namely *A. compressus*, *E. indica*, and *E. colona* (Listihani et al., 2023).

Rice ragged stunt virus (RRSV) belongs to the family *Reoviridae*, genus *Oryzavirus*. The virus has polyhedral particles with a diameter of 50–70 nm. Zhang et al. (2018) reported the rice ragged stunt disease is caused by a round-shaped virus 60 nm in size. RRSV can cause stunted growth, unevenly turned leaf edges, twisted or serrated leaves, swollen leaf blades, the formation of pale yellow to brown ulcers, entwining leaves, incomplete panicle formation, and empty grains. RRSV was first reported in Indonesia in 1976 in the Pandeglang region, West Java (Hibino et al., 1977). A year after, this virus was reported in North Cotabato, Mindanao, the Philippines. Today, rice ragged stunt disease is reported to have spread in Asian countries, including China, India, Japan, Malaysia, the Philippines, Sri Lanka, Taiwan, and Thailand (Nguyen et al., 2015; Lacombe et al., 2021; Phatthalung & Tangkananond, 2022). According to Hibino et al. (1977), in a high-intensity infection, the rice ragged stunt disease can cause up to 50% production loss. In India, the loss reached 80–100%. A survey conducted in Indonesia

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showed that if 34–56% infection occurs, the harvest yield loss will reach 53–82%.

RGSV is a part of family *Bunyaviridae*, genus *Tenuivirus* (Satoh et al., 2013). The RGSV genome organization is formed by six RNA segments that are entirely single-stranded ambisense RNA molecules (Ta et al. 2013). RGSV particles are pleomorphic, appearing like thin filaments or twisting filaments and often form spiral configurations (Phatthalung & Tangkananond, 2022). Du et al. (2005) reported that, in 2000, Vietnam suffered a considerable economic loss due to rice grassy stunt disease, rice ragged stunt disease, and direct damage from brown planthopper infestation. The symptoms of plants attacked by rice stunt disease are stunted growth, excessive tillering, pale green to yellow leaves, yellow to orange narrow leaves, and narrow leaves with small rust patches (Helina et al., 2018).

The diagnosis of stunt disease cannot be made only based on solely symptoms, as the manifesting symptoms are diverse and similar to those in nutritional deficit and drought. The use of molecular techniques in detecting stunt diseases has been frequently reported, and one such method is the polymerase chain reaction (PCR) method (Uehara-Ichiki et al., 2013). Genetic diversity analysis of rice viruses in Asia has been reported by several researchers (Huang et al., 2015; Suprihanto et al., 2015; Sutrawati et al., 2021; Jing et al., 2022). However, the analysis of genetic variation in rice stunt viruses is still limited. Thus, this research aims to determine the disease severity and genetic diversity of stunt viruses in rice crops from Gianyar, Bali. This information is important because there is no existing data on the disease severity and molecular characteristics of RRSV and RGSV Bali isolates.

MATERIALS AND METHODS

Research Site. This research was conducted at rice plantations in Gianyar Regency and the Plant Disease Laboratory, Faculty of Agriculture, Udayana University.

The Rice Stunt Disease Incidence and Severity Field Observation. The observation was performed in seven districts in Gianyar Regency, Bali, namely Blahbatuh, Gianyar, Payangan, Sukawati, Tampaksiring, Tegallalang, and Ubud. The observed parameters include disease incidence and severity. Disease incidence is calculated using the equation below (Listihani et al., 2020):

$$DI = \frac{n}{N} \times 100\%$$

DI = Disease incidence (%);
n = Number of plants with Rice stunt virus symptoms;
N = Number of plants observed.

The disease symptom assessment is performed by assessing visual symptom variations found in the field, including symptom scoring (0–4) (Table 1). The disease severity was calculated according to the following equation (Listihani et al., 2020)):

$$DS = \frac{\sum_{i-1}^{n} (n_i \times v_i)}{N \times Z} \times 100\%$$

DS = Disease severity (%);
n = Number of plants showing certain score;
v = Score i;
Z = Highest score;
N = The number of rice hills observed.

Virus Detection by Reverse Transcription Polymerase Chain Reaction (RT-PCR). Samples were collected from seven districts namely Gianyar Regency, Bali were Blahbatuh, Gianyar, Payangan, Sukawati, Tampaksiring, Tegallalang, and Ubud. A total of 20 samples were taken from each district,

Table 1. Category of the disease severity due to infection of stunt virus in rice cultivations

Category	Score	Description
Healthy	0	No definite symptoms,
Mild	1	Slightly shortened, sometimes with serrated edges, panicles yielded are not as much as healthy plants,
Moderate	2	A bit stunting, a little number of tillering, grows stiff and short, leaves are in dark green color withserrated and twisted edges, leaf blades and sheaths develop swelling, delayed flowering and emptygrains,
Severe	3	Stunting, very few number of tillering, grows stiff and short, narrow leaves, serrated edges with a twist atthe tip, leaf blades and sheaths swelling, partially exerted panicles, malformed panicles, empty grains ordoes not even yield panicles,
Crop failure	4	Stunting, little number of tillering, grows stiff and short, narrow and yellow leaves with mottled spots, does not yield panicles.

resulting in a total of 140 samples in Gianyar Regency. A positive sample, based on the results of RT-PCR with thick DNA bands, was used for sequencing analysis. The detection stages consisted of total RNA extraction, cDNA synthesis, and target DNA amplification.

The total RNA extraction was performed by the Cetyl Trimethyl Ammonium Bromide (CTAB) method (Doyle & Doyle, 1990). The cDNA synthesis utilized total RNA as the template in the reverse transcription reaction (reverse transcription/RT). The RT reaction was carried out in a total volume of 10.00 µL, comprising of 2.00 µL buffer RT, 1.00 µL dNTP 10 mM, 1.00 µL DTT 50 mM, 0.50 µL RNAse Inhibitor (Thermo Scientific, US), 0.50 µL M-MuLV (Thermo Scientific, US), 2.00 µL free nuclease H2O, 1.00 µL Oligo d(T) 10 mM, and 2.00 µL total RNA.

The cDNA synthesis stage began by mixing total RNA with Oligo d(T). The mixture was then incubated in a water bath for 5 min at 65 ºC and immediately cooled by placing the microtube in ice. Other reaction ingredients were added to the microtube, which was then centrifuged for 1 min at 1000 rpm. The microtube was incubated at 42 ºC for 60 min and then at 70 ºC for 5 min for enzyme inactivation.

The target DNA amplification was performed at a total volume of 25.00 µL, comprising 1.00 µL cDNA, 1.00 µL primer F 10 µM, 1.00 µL primer R 10 µM, 12.50 µL GTG Master mix, and 9.50 µL nuclease-free water. The primers used for parsial coat protein (CP) gene of RGSV amplifications were (F:5'-GGCTTATGATAGTCTGTGATTTG-3'/ R:5'GTGTAAGAT GGGGTAAAGTGCA-3') with target amplicons ± 450 bp. The parsial coat protein (CP) gene of RRSV was amplified using the RRSV specific primers (F3:5'-GACTAGGGATGTGCGTTC-3'/B:5'-TGTAATCGACGTTCGCTC-3') with target amplicons ± 210 bp.

The cDNA amplification began with a pre-denaturation stage at 94 ºC for 5 min for one cycle, followed by amplification for 35 cycles. This included a denaturation stage at 94 ºC for one min, primer attachment for 15 sec at 50 ºC, new strand elongation at 72 ºC for one min, and concluded with one final elongation cycle at 72 ºC for 7 min.

The RT-PCR Result Visualization. The DNA amplification product was separated on a 1% agarose gel in 0.5× Tris-Borate EDTA (TBE) at 50 volts for 50 min. Subsequently, the agarose gel was immersed in a 0.1% ethidium bromide solution for 5 min. Visualization was carried out under a UV transilluminator, and the results were documented using a digital camera.

Nucleotide Sequence Analysis. The DNA amplification product was sequenced at First Base, Malaysia. The DNA sequence was analyzed using the Basic Local Alignment Search Tool (BLAST) on the National Center for Biotechnology Information website (www.ncbi.nlm.nih.gov) to compare the target virus sequence with virus nucleotide sequences from other countries registered in the GenBank database, as implemented in the program BioEdit v7.05. Phylogenetic analysis was constructed using MEGA v6.0 software with the neighbor-joining algorithm and a bootstrap value of 1000 repetitions (Tamura et al., 2013).

RESULTS AND DISCUSSION

The stunt disease symptoms started to appear at 2 weeks after planting (WAP) and continued to increase until 7 WAP (Figure 1A and B). The observed symptoms included those of ragged stunt disease, grassy stunt disease, and a mix of symptoms from both diseases (Figure 1C, D, E). Identifying stunt disease symptoms in the early growth stage is challenging. The inhibition of plant height was more noticeable in grassy stunt disease than in ragged stunt disease (Figure 1C and D). In plants infected with grassy stunt disease, the height inhibition was evident during the vegetative phase with excessive tillering, while in plants infected with ragged stunt disease, the reduction in plant size became apparent upon entering the generative phase. Double infection was also observed in rice crops in the field, specifically the double infection of ragged stunt disease and grassy stunt disease in rice crops in the Tampaksiring District.

The symptoms of double infection with ragged stunt disease and grassy stunt disease include twisted leaf parts with yellowing on various sections, along with several leaf tips and leaves becoming narrow (Figure 1E). According to Du et al. (2005), plants infected by both RRSV and RGSV exhibit more severe stunting compared to single infections. Generally, RRSV symptoms manifest first with dark green tillering, thinner and straighter than normal with small tillers, followed by RGSV symptoms where young leaves turn browner, and there is usually yellowing of old leaves. The symptoms of double infections by both viruses have also been reported in volunteer plants (Dini et al., 2015). Volunteer plants could become infected by the virus, so clearing the field of volunteer plants is necessary to eliminate the inoculum source

and prevent a broader viral spread in the next planting season (Helina et al., 2018). Sanitation measures can also be implemented before and during the planting season to manage weeds in the field, as RGSV and RRSV have been reported to infect weeds around rice fields (Listihani et al., 2023).

There are clear differences between plants suffering from ragged stunt disease and grassy stunt disease. Plants infected by the ragged stunt disease virus exhibit more varied symptoms compared to grassy stunt disease virus infection in rice crops (Figure 1). The leaves remain dark ground, the tips of the leaves curl, and in several plants, symptoms appear in leaves in the form of rips and wrinkles, making the leaf surface appear rough. Swelling could be found on the underside of the leaves. Rice crops with ragged stunt disease symptoms show unique features, including stunting, leaves turning dark with serrated edges or curly tips, swollen leaf blades, or bumps on the underside of the leaves or the outer side of the leaf sheaths. These bumps are due to hyperplasia and hypertrophy of phloem tissue (Muduli et al., 2021).

The highest incidence of stunt disease was found in three districts: Ubud, Tampaksiring, and Payangan, with rates exceeding 44% (Table 2). Stunt disease incidences in Blahbatuh and Gianyar District are considered low, being less than 10%. This might be attributed to the population fluctuation of its vector, the brown planthopper, in the field. Such fluctuations could arise due to environmental factors influencing the insect vector population (Triwidodo & Listihani, 2020; Listihani et al., 2022a; Temaja et al., 2022),

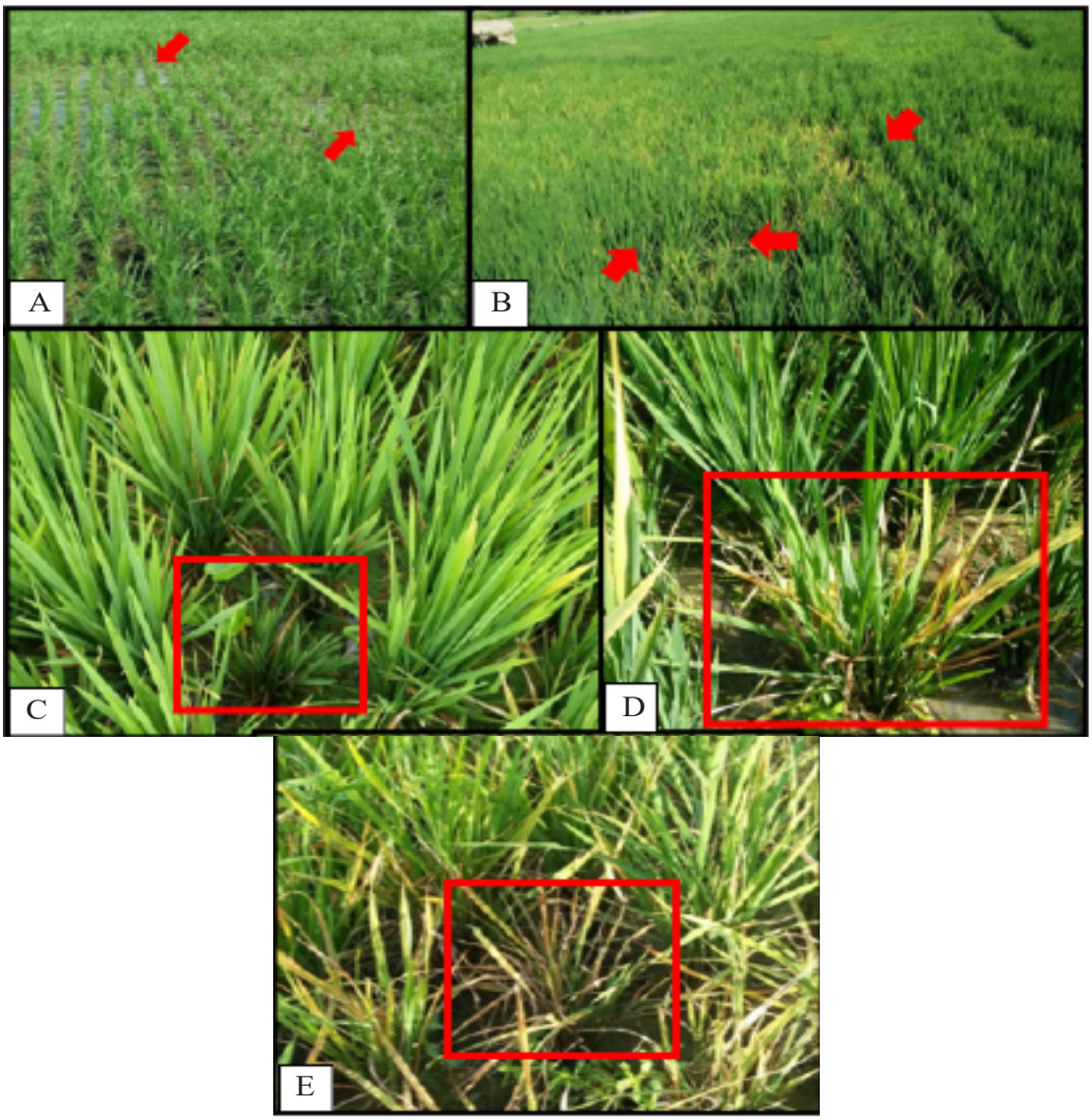

Figure 1. Symptoms variation of rice stunt virus in Gianyar Regency, Bali. A= Vegetative phase of plants infected with stunt virus; B= Generative phase of plants infected with stunt virus; C= Rice infected with RRSV; D= Rice infected with RGSV; E= Mix infection of RRSV and RGSV.

as well as the cultivation pattern influencing disease distribution (Listihani et al., 2023).

The rice cultivation pattern in Gianyar Regency is considered monocultural with an unsynchronized planting period. The high population of the vector *Nilaparvata lugens* in the field leads to a high incidence of stunt disease (Listihani et al., 2022a). This is due to the high percentage of viruliferous *N. lugens* accelerating the spread of RGSV and RRSV in the field. Dini et al. (2015) showed that a high stunt disease virus incidence in the field is due to stunt viruses having the ability to cause disease faster than the tungro viruses. Disease incidence is influenced by the differences in vectors transmitting the virus semi-persistently and persistently to plants (Lu et al., 2019; Listihani et al., 2020; Listihani et al., 2022b; Listihani et al., 2022c; Selangga & Listihani, 2022; Selangga et al., 2022; Selangga et al., 2023). Stunt viruses are transmitted in a persistent propagative fashion to the plants by the vector *N. lugens*, making the vectors highly capable of transmitting the virus and causing a high incidence of stunt disease in the field. The incidence of rice stunt diseases in Bali is still sporadic but requires attention as it correlates with the population dynamics of its vector insect, the brown planthopper.

The stunt disease severity in Gianyar Regency ranges from 18.84% to 60.82% (Table 2). The most severe stunt disease occurs in Tampaksiring District (60.82%), while the lowest occurs in Gianyar District (18.84%). Disease severity is influenced by plant resistance, pathogen virulence, plant cultivation method, and environmental condition that supports disease occurrence in the field. Listihani et al. (2022a) reported that IR-64 and Ciherang rice cultivars are vulnerable to *N. lugens* infestations, which is the vector of rice stunt viruses. Moreover, disease severity is also influenced by other pathogens or pests that infect simultaneously with stunt viruses on the same plant. The research results from the field also found that there are non-vector insects (green leafhoppers and white-backed planthoppers), rice blast disease, and tungro virus in rice plants that can increase plant vulnerability to stunt disease infection.

The disease severity level is also determined by virus virulence and the host plant's response. Listihani et al. (2023) stated that the disease incidence and severity are a result of the interaction of several factors, including virus isolate/strain, rice cultivar, and the environmental conditions of cultivation, which include the presence of the insect vector. Moreover, Helina et al. (2018) reported that rice cultivars could exhibit the same resistance response against a viral strain despite having different resistance genes.

The result of this research showed that IR-64 and Ciherang cultivars are vulnerable to RGSV and RRSV stunt virus infection. This research aligns with Suprihanto et al. (2015), who stated that IR-64, Ciherang, and Inpari 1 are cultivars vulnerable to stunt virus infection, while Mentik Wangi and Tetep are cultivars resistant to stunt virus infection. Other than causing lower plant height and changing leaf shapes and colors, stunt virus infection in rice crops can also influence the formation and ripening process of grains (Dini et al., 2015). This could be observed through direct comparisons between healthy plants and several plants infected by the stunt virus in Gianyar Regency. Although the harvest loss due to virus infection could not be quantified, qualitatively, it could be observed that healthy plants produced many ripe grains that could be harvested, whereas rice crops infected by stunt virus showed less grain formation and were still within the milk stage when they were supposed to be ready for harvest. High disease severity also caused rice crops not to produce panicles and grains.

Helina et al. (2020) stated that rice production evidently drops the higher the planthopper infestation, and the earlier the planthopper attacks, the higher the planthopper population. Yield loss due to disease in rice is also influenced by the infected plants' stadia (age). Infection during 2–12 weeks after planting can cause yield loss between 23.86–85.42% (Chen et al., 2019). This shows that the presence of stunt virus carried by

Table 2. Disease incidence and severity of rice stunt virus in Bali, Indonesia

Location	Rice cultivars	Disease incidence (%)	Disease severity (%)
Blahbatuh	Ciherang	9.22	21.89
Gianyar	IR-64	8.17	18.84
Payangan	Ciherang	48.31	54.92
Sukawati	IR-64	13.23	26.71
Tampaksiring	Ciherang	45.92	60.82
Tegallalang	Ciherang	23.27	32.18
Ubud	IR-64	44.65	52.72

brown planthoppers causes a greater setback in panicle growth and fruit formation in rice crops.

Stunt virus detection was performed for each sample obtained from field observation. Virus detection from plant samples obtained from the field confirmed the symptoms of the infecting virus type. RRSV DNA strands ± 210 bp in length were amplified from samples showing the symptoms of ragged stunt disease, while RGSV DNA strands ± 450 bp in length were amplified from samples showing grassy stunt disease symptoms. Two DNA strands, ± 210 bp and ± 450 bp each, were obtained from samples showing mixed symptoms of grassy stunt disease and ragged stunt disease in Tampaksiring District (Figure 2). This showed that rice crops in Gianyar Regency were positive for grassy stunt virus and ragged stunt virus infection.

Molecular detection of RRSV dan RGSV has been reported by Plant Protection Research Institute/ PPRI (2012) and Dini et al. (2015). Specific primers used by PPRI to amplify RRSV and RGSV with the PCR method are different from the primers used in this research. The RRSV amplification was performed using primer pairs RRSV-F3/ RRSV-R3 or RRSV-F9/ RRSV-R9 with amplicon targets being 825 pb and 1110 pb consecutively, while RGSV amplification used primer pair RGSV-P5F/RGSV-P5R with an amplicon target being 885 pb. The amplicon obtained was larger compared to the target amplicon used in this research, where they were 210 bp for RRSV and 450 bp for RGSV. On another note, Helina et al. (2018) used the same primers in this research: RGSV F1/R primer and RRSV F3/R3 primer.

The RRSV and RGSV amplification product DNA sequencing produced sequences 210 bp and 450 bp in length, respectively (Figure 2). The RGSV sequence analysis showed that Indonesian isolates (Ubud and Payangan) have high homology with isolates from Vietnam (FM995505), Kamboja (KF438708), and the Philippines (KF438684) with >97% and the lowest homology with Cambodian isolate (KF438744) with 96.1% (Figure 3). The RRSV sequence analysis showed that Indonesian isolates (Payangan and Ubud) have high homology with isolates from Vietnam and Thailand with >98%, and the lowest homology with the isolate from China with 95.3% (Figure 4).

The RGSV phylogenetic tree formed two groups: Group 1 (Indonesia, Vietnam, Kamboja, and the Philippines) and Group 2 (Cambodia). The RRSV phylogenetic tree formed three groups: Group 1 (Indonesia and Vietnam), Group 2 (Thailand), and Group 3 (China). The Indonesian isolates of RGSV and RRSV showed a close relationship with Asian isolates.

Rice virus nucleotide analyses have been frequently reported for other viruses in rice crops (Zhao et al., 2017; Kannan et al., 2019), but are still limited for stunt viruses. The search for RGSV and RRSV sequences on GenBank managed to obtain six and three virus isolates, respectively for each. Upadhyaya (1995) reported that Thailand RRSV isolates have high homology with India RRSV isolates, 94.6% and 99.4%, respectively for nucleotide and amino acid sequences. An RGSV sequence analysis result was reported by Lianhui and Qiying (2003), which stated RGSV-SX isolates have a homology level of 99.1% and 96.2% respectively for nucleotide sequence with RGSV-IR and RGSV-SC isolates; while the amino acid

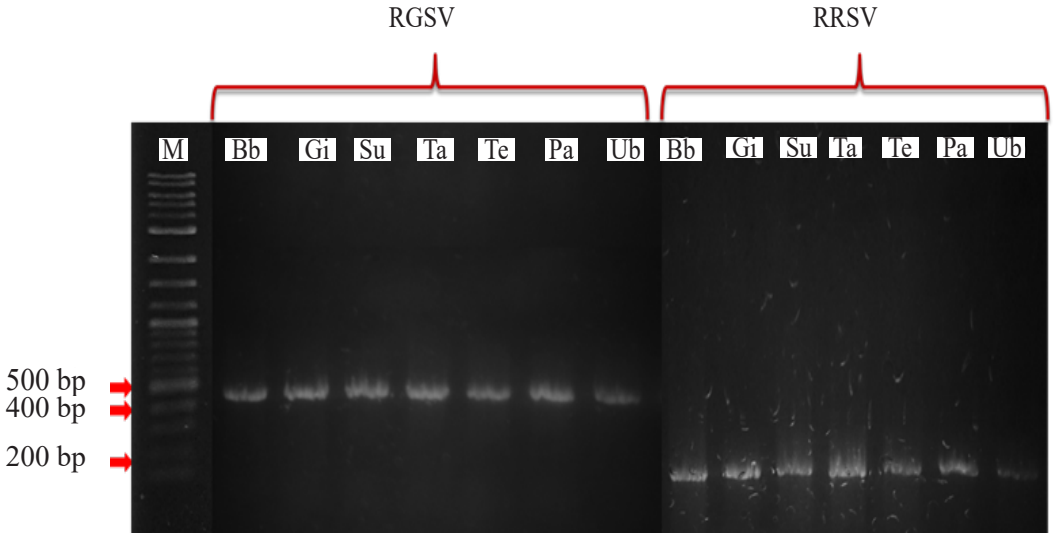

Figure 2. Visualization of DNA amplification of RGSV F1/R and RRSV F3/B3 with the size ± 450 bp and ± 210 bp, respectively; Bb= Blahbatuh; Gi= Gianyar; Su= Sukawati; Ta= Tampaksiring; Te= Tegallalang; Pa= Payangan; Ub= Ubud; M= DNA marker 1 kb (Thermo Scientific).

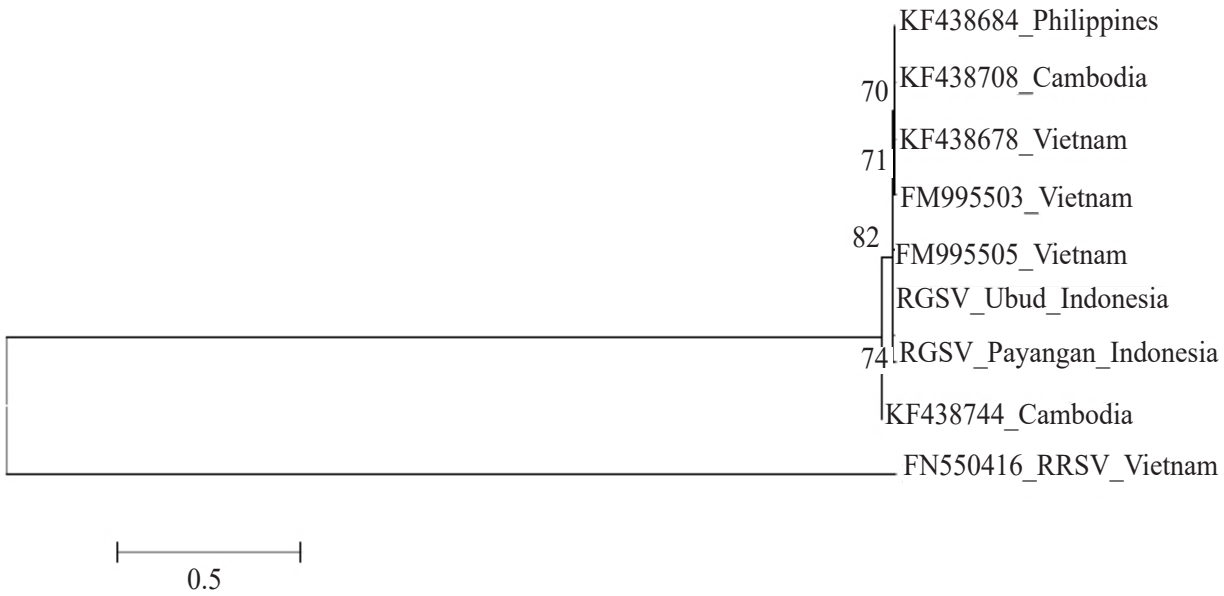

Figure 3. Phylogeny tree of nucleotide sequences of RGSV Gianyar isolates using MEGA 6.0 (Neighbour Joining with bootstrap 1000×). RRSV from Vietnam is used as out groups. The highlight mark is a RGSV isolates from Gianyar Regency.

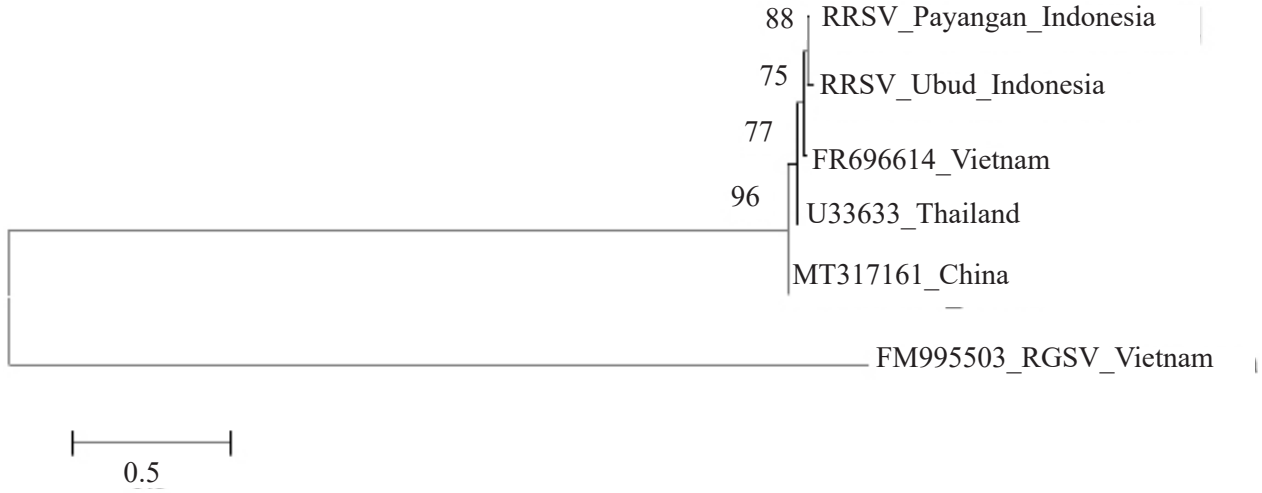

Figure 4. Phylogeny tree of nucleotide sequences of RRSV Gianyar isolates using MEGA 6.0 (Neighbour Joining with bootstrap 1000×). RGSV from Vietnam is used as out groups. The highlight mark is a RRSV isolates from Gianyar Regency.

sequence homology was 98.4% and 96.4%. According to King et al. (2012), a virus is considered to have a close relationship if it shows a nucleotide sequence homology of >89%. Thus, the rice virus isolates in Southeast Asia with very high homology are assumed to have close genetic relationships.

CONCLUSION

Stunt disease caused by RRSV and RGSV was found infecting rice crops in seven districts in Gianya Regency, Bali. The incidence rate for stunt disease in Gianyar Regency was highest in Payangan district while the disease severity level was highest in Tampaksiring District. IR-64 and Ciherang cultivars are vulnerable to stunt disease infection from RGSV and RRSV. The nucleotide homology for RGSV and RRSV isolates from Gianyar, Indonesia, was highest with Vietnam isolates and forms the same cluster in the phylogenetic tree analysis. Nucleotide sequence analysis showed a relatively close genetic relationship between RRSV and RGSV Indonesian isolates with other isolates from Asia.

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AUTHORS' CONTRIBUTIONS

LL considered and planned the experiment. LL, DGWS, KAY, IGADY, and PEPA carried out the survey in the fields, sampling, and observation of disease incidence and severity in the fields. LL and DGWS performed molecular identification work and bioinformatica analysis. All the authors have prepared the manuscript and have read and approved the final manuscript.

COMPETING INTEREST

We declare there are no relevant financial or nonfinancial competing interests to report.

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