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Biological dan Molecular Characterization of Papaya Ringspot Virus from Bogor District, Indonesia

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Abstract

Management of PRSV using cross-protection and transgenic plants has been hampered due to varying PRSV gene sequences. Therefore, the characterization of new PRSV isolates could help design the region needed for region-specific management practices. The study aimed to characterize the typical PRSV isolates found in Bogor biologically and molecularly. The study was conducted in 2 stages: host range study of 2 isolates (Sukaraja and Cijeruk) on five species with six plants for replication, and RT-PCR analysis amplified coat protein (CP) region using PRSV326 and PRSV800 primer pair. The results showed that Sukaraja isolate produced systemic symptoms in papaya (*Carica papaya* L) cv. Merah Delima in the form of leaf lamina becoming pale and wilting, leaf malformation, open veins, and striped patterns on the leaves, while the Cijeruk isolate causes symptoms of leaf blistering, mosaicism, leaf malformation, wilted lamina, and striped patterns on the leaves. These two isolates did not cause symptoms on eggplant (*Solanum melongena*) and chickpea (*Vigna unguiculata*) but produced striped leaf patterns and pale lamina symptoms on bitter melon (*Momordica charantia*) and melon (*Cucumis melo*) leaves. RT-PCR analysis was able to amplify ±475 bp of DNA. The two DNA isolates had a homology percentage of 97.7% with PRSV isolates from Thailand, ranging between 93.07-99.68% with the Kulon Progo and Nganjuk isolates. Based on phylogenetic analysis, the Cijeruk isolate was in the same branch as the Indonesian isolate, while the Sukaraja isolate was a separate branch and closely related to isolates from Oklahoma and Malaysia.

Keywords: Homology, host, PRSV, PCR Analysis, symptom

Introduction

Papaya (*C. papaya* L) originated from Mexico but nowadays it has spread and become an essential horticultural crop in Indonesia. According to Faostat (2022), Indonesia is the third-largest papaya-producing country in the world. Apart from the flesh, it is also rich in vitamin C, and the skin and seeds of the papaya fruit contain

phenols and flavonoids, which are excellent sources of antioxidants (Insanu et al., 2022).

Papaya production in Indonesia fluctuates yearly, and the main contributor to the fluctuations is ring spot disease, a new disease that causes significant damage to papaya production in Indonesia. This disease is caused by the *Papaya ringspot virus* (PRSV), a member of the Potyvirus genus in the Potyvirus family (Purcifull et al., 1984). PRSV is a single RNA virus in the form of a filament with a particle size of 700-900 nm. PRSV, which is transmitted semi-persistently by aphid vectors (Basavaraj et al., 2019), consists of two types: type P infects papaya and *Cucurbitaceae*, and type W only infects *Cucurbitaceae* (Desbiez et al., 2020).

Papaya ringspot virus (PRSV) disease was first detected in Mexico in 1975 (Noa-Carazzana et al., 2006); furthermore, it spread to other papaya-producing countries. In Indonesia, PRSV was first found in Aceh in 2012 (Hidayat et al., 2012; Farida et al., 2022) then spread rapidly to North Sumatra, West Java, East Java, and Bali with severe damage. The PRSV-P pathogen is reported to produce diverse symptoms (Sosol-Reyes et al., 2020); it causes vein-clearing and yellowing symptoms on young leaves and curling, deformation, and stunting on papaya leaves in India (Chalak et al., 2017). From several reports of PRSV disease incidents in Indonesia, generally, papayas infected with PRSV-P appear to have mosaic symptoms, transparent veins on the leaves, ring spots on the fruit, and oily spots on the stems (Farida et al., 2022; Riska et al., 2023).

PRSV isolates from Indonesia, especially the Java region, are thought to have entered Thailand—a curly disease attack in Sukaraja Bogor was reported in 2014 (Yoeshinanda and Widodo, 2014), but the cause is unknown. Yoeshinanda and Widodo (2014) stated that papaya cultivation has spread widely in the West Java region since 2011. In another location in Bogor, Lestari (2014) stated that Papaya ringspot disease caused by PRSV had attacked papaya in that area. PRSV attacks were reported in 2022 in Bogor, specifically in the Darmaga and Tanah Sereal subdistricts (Farida et al., 2022). Based on molecular identification, isolates from Java (Bogor, Kebumen, Purworejo, Bantul, and Nganjuk) and Bali were included in the PRSV-P group from Thailand (Farida et al., 2022). At this time, mobility of exchange and demand for plant seeds is accessible from one area to another and is one of the triggers for the spread of Papaya ringspot disease. Apart from that, this mobility also impacts changes in the virus's virulence and genotype variations due to gene recombination or genetic drift. So, characterization of virus isolates from various locations and different times is necessary to understand the pathotype of the virus and determine appropriate, efficient, and effective PRSV control measures. The research aimed to characterize the typical symptoms of PRSV biologically and molecularly in two papaya plantations in Bogor, West Java, Indonesia.

Methods

The research was carried out in farmers' seed collection in Solok, West Sumatra (0o46’32.52846”S100o37’37.17179 ”E) and the Seed Quality Testing Laboratory, Center for Standardization of Agricultural Instruments for fruit crops, Solok, West Sumatra, Indonesia, from February to May 2023.

Isolates Collection

There were six samples of leaves and petioles were collected from papaya plantations that attacked by a disease similar to the typical symptoms of Papaya ringspot disease, such as oily transparent spots on the stems, fruit, and stalks, as well as mosaics on the leaves in Sukaraja and Cijeruk, Bogor

Regency, West Java, Indonesia, in February 2023. Details of the location are presented in Table 1. Sampling was carried out from 1 ha papaya plantation. Samples were taken from the third leaf of the shoot and had severe symptoms. Samples were prepared by placing 2-3 leaves in a straw envelope and storing them in a plastic box containing 1/3 of the leaves with silica gel.

Table 1. Sampling location, affected varieties, and extent of disease attack in Bogor, West Java, Indonesia

Isolate Code	Location	Coordinate	Variety	Land area	Attack intensity (%)
Cijeruk	Sukaharja, Cijeruk, Bogor	6°40'19.8" S106°45'49.6" E	California	>1 ha	±100
Sukaraja	Sukaraja, Pasir Jambu, Sukaraja, Bogor	6°31'57.3 " S106°49'00.9" E	Calina	0.25 ha	±20

Plant Preparation

The source of healthy papaya seeds (*C. papaya*) var MD. is two-month-old MD (5-6 leaves, 15 cm high) was obtained from the house nursery of Agency for Standardization of Agricultural Instruments for fruit crops, Solok, West Sumatra, Indonesia

Virus Extraction, and Virus Inoculation

The source of virus inoculum were fresh infected papaya leaf samples and silica gel preserved samples. A total of 0.2 g of selected leaf samples were ground with a sterile mortar (1:20 w/v) containing phosphate buffer solution (0.4 g of NaH2PO4; 0.9 g of Na2HPO4, H2O, 500 ml of teryl distilled water, pH 7.6) and the virus sap was inoculated by wiping two leaves (the 3rd and 4th leaves from the shoot) which had previously been sprayed with carborundum mesh of 600 nm. After inoculating the virus sap, the inoculated part of the leaf was rinsed with running water to remove any virus sap attached from the plant's surface. Plants were then maintained in wooden boxes with chiffon walls in daily temperatures ranging from 30 ± 2°C and normal lighting for 12 hours.

Total RNA Extraction and Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR).

Viral RNA was extracted from 0.2 g of papaya leaves (2 samples) using a total RNA kit (Tiagen Biotech, Beijing). Single-strand cDNA was hybridized with oligo-dT primer and random primer using Rever-Tra Ace (Toyobo, Osaka, Japan) at 42°C for 30 minutes and 99°C for 5 minutes (Riska et al., 2019). The PCR amplification process was carried out using PRSV-specific primers (PRSV326: '5-TCGTGCCACTCAATCACAAT-3') and (PRSV800: 5'-GTTACTGACACTGCC GTCCA-3') which target the coat protein (CP) at a size of 475 bp (Mohammed et al., 2012).

PCR amplification using an Eppendorf® machine (Mastercycler® Nexus Thermal Cyclers, Hamburg, Germany) began with predenaturation at 94 °C for 1 minute, followed by 30 cycles of denaturation for 30oC at 94 °C, primer attachment process at 55 °C for 15 seconds, elongation at 72 °C for 1 minute and final elongation for 10 minutes. Each DNA product was separated with 1.2% agarose gel through an electrophoresis stage (100 v for 30 minutes). The 475 bp DNA

product was purified and sequenced using the First Base technique (Genetic Science co.ltd. Jakarta). Nucleotide sequence sequencing and pairwise comparison of nucleotide identities were calculated using the Bioedit software and EMBOSS pairwise alignment algorithms program (http://www.ebi.ac.uk/Tools/psa/emboss_needle).

Host Range Test

In this study, five plant species from four families were used; papaya, eggplant (*S. melongena*) var. local, chickpea (*V. unguiculata*) var. Logawa, bitter melon (*M. charantia*) var. Hainan F1, and melon (*C. melo*) var. Jumbo F1. A total of ±3 seeds for each plant were germinated in polybags containing a mixture of soil, manure, and rice husks (2:1:1 v/v/v) and maintained for ±1 month (After the seeds developed, one seed that grew was left rod for testing). Papaya seeds var. MD as a control plant was obtained from the seed house of Center for Standardization of Agricultural Instruments for fruit crops, Solok, West Sumatra, Indonesia. The papaya used was a plant approximately two months old with a size of ± 15 cm and 5-6 leaves. Daily temperature during seed propagation is 30 ± 2°C, and normal lighting is 12 hours/day.

Virus inoculation on plants was carried out like the method for multiplying the virus inoculum source, referring to Riska et al. (2023). The plant leaves used as an inoculum source were the third from MD papaya shoots aged one month after artificial inoculation. Virus sap in a phosphate buffer solution was applied to healthy host plants' 3rd and 4th leaves. Six plants of each plant were inoculated. Plants after inoculation were maintained for one month in 30 ± 2°C, and normal lighting is 12 hours/day.

Variables Observed

Symptoms of viral infection from the field

Symptoms in the field were observed morphologically/macroscopically, then compared with the symptoms reported by .... (literature)

Symptoms of inoculated virus infection on some plants

Symptoms were measured by looking at the symptoms that appeared on the plants, such as mosaics, leaf malformations, blanching of the lamina, and yellowing of the leaves observed for 30 days.

Recombinant and phylogenetic analysis

The potential for recombination of the two isolates and their cognate genes was analyzed using the RDP, GENECONV, BOOTSCAN, MAXCHI, CHIMAERA, SISCAN, and 3SEQ methods applied to the RDP4 program (Martin et al., 2015). For each recombination cut point, a Bonferroni-corrected P value was calculated (with a cutoff of $P<0.05$). Next, a phylogenetic tree was constructed using MEGA 11.0 software (Tamura et al., 2021).

Results

Symptoms of a Virus Attack in the Field

In this study, symptoms of papaya plants found in Sukaraja and Cijeruk farmers' gardens visually showed the typical characteristics of PRSV infection. Papaya plants attacked by the disease in Sukaraja showed yellow mosaics, leaf malformations, transparent veins, and oily spots on the fruit, fruit stalks, and stems (Figure 1a). Symptoms of papaya plants found in Cijeruk were blistered leaves and oily ring spots on the fruit, stalks, and stems (Figure 1b). Symptoms of this disease were found to be evenly distributed and severe in gardens covering more than 1 ha.

Host range test

The host range test (Table 2, Figure 2) carried out during one month of observation showed symptoms of attack by both isolates on papaya (*C. papaya* L.) plants cv. MD looks different morphologically. Sap virus originating from isolate

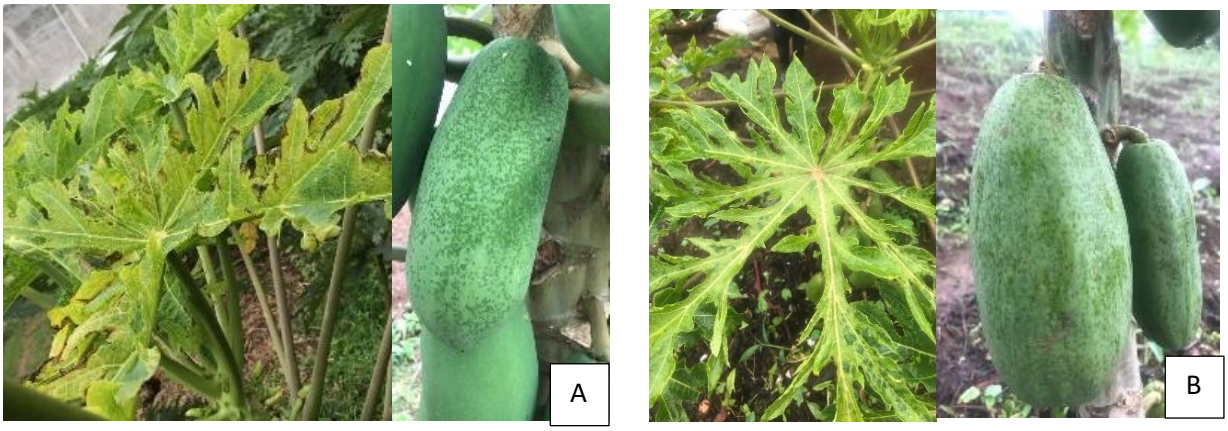

Figure 1. Characteristics of papaya ring spot virus: A. Yellowing, mosaic, stunted plants, ring and oily spots on fruit induced by pathogens found in Sukaraja, B. Blistered leaves, ring spots, and oily spots on fruit caused by pathogens in Cijeruk.

from Cijeruk caused more severe infections in papaya seeds than Sukaraja isolates. The Sukaraja isolate causes symptoms of pale and wilted leaf lamina, leaf malformations, transparent veins, and striped patterns on the leaves, while the Cijeruk isolate causes symptoms of leaf blisters, mosaics, leaf malformations, wilting of the lamina, and striped patterns on the leaves. Both isolates caused uniform symptoms, namely leaves forming a striped pattern and symptoms of lamina turning pale on bitter melon (*M. charantia*) and melon (*C. melo*) leaves. Both isolates did not cause symptoms in eggplant (*S. melongena*) and beans (*V. unguiculata*) plants.

Table 2. Host range and symptoms caused by Sukaraja and Cijeruk isolates on inoculated leaves and upper leaves after one month of inoculation.

	Symptoms Description	
Host	**Sukaraja**	**Cijeruk**
Papaya (*C. papaya* L)	Lamina was pale, wilted; leaves became malformations, transparent veins, and mottled patterns	scalded leaves, wilted lamina, mosaic, leaves became malformations, and mottled patterns
Egg plant (*S. melongena*)	without symptoms	without symptoms
Bitter melon (*M. charantia*)	mottled pattern on the leaves	mottled pattern on the leaves
Melon (*C. melo*)	mottled pattern on the leaves	Lamina was pale
Chickpea (*V. unguiculata*)	without symptoms	without symptoms

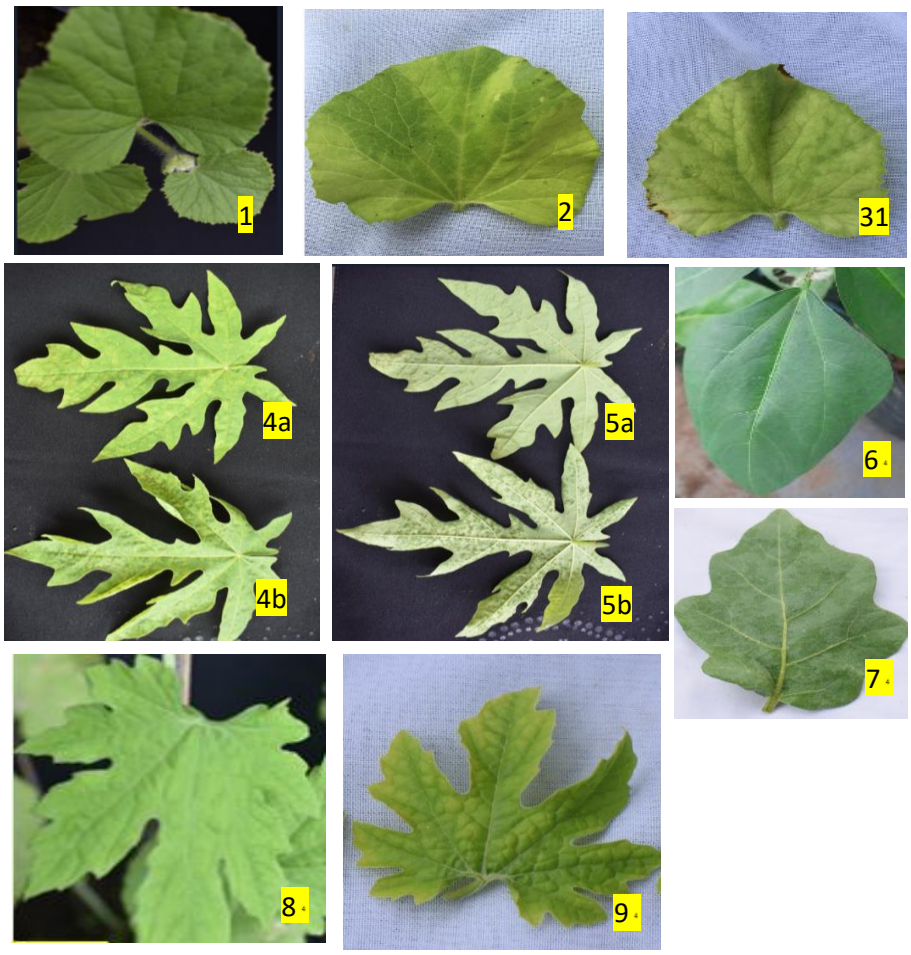

Figure 2. Symptoms of virus attack and healthy symptoms on host plants infested with Sukaraja and Cijeruk isolates: 1. Melon (*C. melo*) is healthy, 2. Melon was infected with the Sukaraja isolate, 3. Melon was infected with the Cijeruk isolate, 4a. The upper surface of papaya leaves var. MD was attacked by the Sukaraja isolate, 4b. The upper surface of papaya leaves var. MD was attacked by the Cijeruk isolate, 5a. The lower surface of papaya leaves var. MD was attacked by the Sukaraja isolate, 5b. Lower surface of papaya leaves var. MD was attacked by the Cijeruk isolate, 6. Chickpea leaves (*V. Unguiculata*) had no symptoms, 7. The leaves of the eggplant (*S. melongena*) had no symptoms, 8. Bitter melon leaves (*M. charantia*) were healthy, 9. Bitter melon leaves (M. charantia) have mosaic symptoms

Recombinant and Phylogenetic analysis

Dari Gambar 3 terlihat sedikit variasi pada runutan DNA dari kedua isolat. Pada isolat Sukaraja, posisi ke-205 adalah basa Timin sedangkan tiga isolat lain adalah Sitosin. Nukleotida di posisi 201 pada isolat Sukaraja, Cijeruk dan Nganjuk adalah Adenin, pada isolat Kulon Progo adalah Timin. Pada posisi basa ke-355, nukleotida pada isolat Sukaraja adalah Timin sedangkan pada tiga isolat lain adalah Sitosin. Kemudian pada posisi 370, Adenin pada isolate Sukaraja, Cijeruk dan Nganjuk sedangkan Guanin pada isolat Kulon Progo.

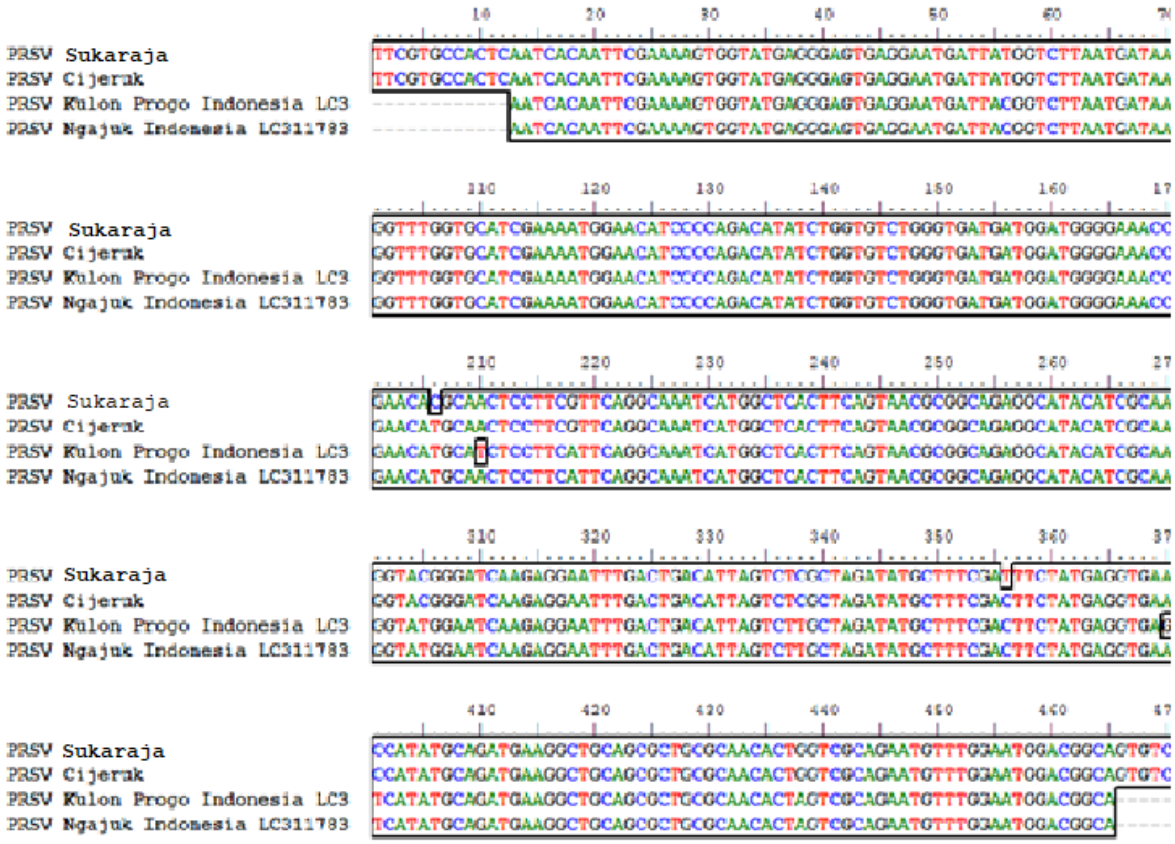

Figure 3. The DNA sequence of the partial coat protein gene of Cijeruk, Sukaraja isolates, and PRSV isolates found in Java (Kulon Progo and Nganjuk isolates).

Analysis results using NCBI BLAST, Isolate of Sukaraja, and Cijeruk ranged from 97.7 to 98.23% identity with PRSV from Thailand (accession AF506899). The total nucleotide sequence of the two isolates aligned/traced with Emboss Needle was 470 bp, with the homology between the two isolates being 99.5%. The homology with other isolates from Java ranged from 93.07 to 99.68%. The Sukaraja isolate showed a lower homology rate than the Java isolate compared to the isolate from Cijeruk. The homology of the Sukaraja isolate with the Kulon Progo and Nganjuk isolates was 93.07 and 93.8%, while with the Cijeruk isolate, it was 99.68 and 97.35%, respectively. The results revealed that the two isolates from Sukaraja and Cijeruk are classified as having a close relationship with isolates previously found in the Java region (Table 3).

Table 3 Percentage of nucleotide sequence homology of the CP gene of PRSV isolates from papaya from Bogor and isolates found in East Java.

Isolat	Homology (%)	
	Cijeruk	Sukaraja
Kulon Progo LC311782	99.68	93.07
Nganjuk LC311783	97.35	93.8
Cijeruk	-	99.5

Recombination analysis of the two isolates with other PRSV isolates using the RDP4 program showed no recombination events, with no recombination events detected in the partial CP of the two isolates. This became the basis for a phylogenetic tree analysis to determine the relationship between the isolates from Bogor and other PRSV isolates using the Neighbor-Joining method in the MEGA 11.0 software.

Figure 4 showed a slight contrast with the results of the homology analysis. The results of the Neighbor-Joining analysis of partial CP sequences of the two isolates from Cijeruk and Sukaraja were grouped at different branches. The Sukaraja isolate formed a single branch but was closely related to the Oklahoma and Malaysian PRSV isolates, followed by the Pakistani and Indian isolates. Meanwhile, the Cijeruk isolate was closely related to the PRSV isolate from Indonesia, Nganjuk, Kulon Progo, Brebes, and Bali isolates. In line with the BLAST analysis, which showed that the Cijeruk isolate had very high genetic similarity to the Thailand isolate, this phylogenetic analysis showed the same thing: the Cijeruk isolate was in the same branch as the Thailand PRSV isolate (accession U14743.1).

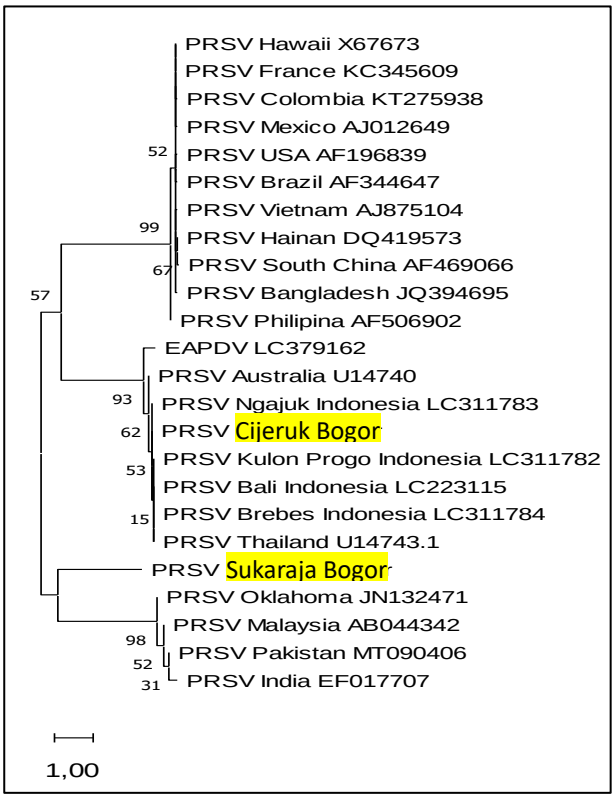

Figure 4. A phylogeny tree of the envelope proteins of two Bogor isolates and 30 world PRSV isolates was built using the neighbor-joining method algorithm. The percentage of isolates that clustered together was measured using 1000x bootstrap. Trace and ponon phylogenies were created using the MEGA.11.0 program

Discussion

Based on the observation conducted in Sukaraja and Cijeruk, papaya plants in this area were attacked by ring spot disease (Figure 1). leaf samples with symptoms of mosaic yellowing and ring and oily spots on the fruit, fruit stalks, and stems are the dominant symptoms and are evenly found in papaya plantations. These symptoms are typical symptoms caused by PRSV. However, from the direct observations and information from farmers, the Calina papaya variety is aged ± 4-5 months in gardens located in Cijeruk, it was accompanied by symptoms of scalded leaves and malformations on the leaves and caused yield losses of up to 100%. These symptoms were slightly different from

those produced by PRSV isolates from Bogor: mosaicism and blanching of the lamina (Harmiyati et al. (2015). Differences in environmental conditions, plant age, and varieties (Harmiyati et al., 2015; Sósol-Reyes et al., 2020) may cause different symptoms and differences in the severity of diseases that appear on plants.

Efforts to determine the distribution of hosts are basically to determine the role and involvement of hosts other than papaya in spreading PRSV disease. This host range test is often an initial clue before further microscopic and molecular identification. In this study, the two isolates produced slightly different symptoms, but visually, the symptoms were very severe on papaya. The Sukaraja isolate appeared to have a dominant yellow mosaic, while the Cijeruk isolate appeared to cause the leaves to blister and change shape (Table 2, Figure 2). Harmiyati et al. (2015) stated that when mechanically inoculated with 5 PRSV isolates, one of the isolates from Bogor, several papaya varieties produced blanched leaves and slight mosaicism with different levels of severity. This showed that the symptoms that appear on plants infected by specific isolates can be visually different. Plant and environmental conditions also influence disease symptoms due to pathogen infestation (Liu et al., 2009). Both isolates caused changes in the leaves of melon and bitter melon plants, namely the blanching of the lamina and the formation of striped patterns on the plant leaves. Coherent with many report that Melon and bitter melon, a group of Cucurbitaceae, can be affected by PRSV isolation (Zhu et al., 2016; Kumar et al., 2021; Riska et al., 2023).

Furthermore, these two isolates did not cause symptoms on eggplant (*S. melongena*) and beans (*V. unguiculata*) (Table 2, Figure 2). The result equivalent with isolates North Sumatera and West Java that cannot produce typical symptom of virus infection on eggplant (*S. melongena*) and beans (*V. unguiculata*) (Riska et al., 2023). From these results, it is currently suspected that the virus that causes disease in papaya plants in Cijeruk and Sukaraja Gardens is PRSV or belongs to the PRSV-P strain. PRSV consists of two strains, namely PRSV-P and PRSV-W. The PRSV-P strain, apart from Caricaceae, can infect the Chenopodiaceae and Cucurbitaceae families (Kumar et al., 2021). Apart from the results of this host range test, Farida et al. (2022) stated that the PRSV strain that spread in Dramaga Bogor is the PRSV-P strain; in fact, this PRSV has been detected in Bogor since 2012, although the exact location is unknown (Harmiyati et al. al., 2015).

Although the host range test is an initial indication for identifying virus species, environmental influences, plant health conditions, or attacks by other pathogens can invalidate identification results based on symptom manifestations. Characterization of the viral CP region is the method most widely used in identification, taxonomic estimation, and phylogenetic studies (Jain et al., 2004). The results of measuring the nucleotide identity of the CP gene showed that the two PRSV isolates in this test belong to the PRSV species. This can be seen from the relatively high percentage of homology between the two isolates and the Thai PRSV isolate, namely more than 93% (Table 3, Figure 3). According to the criteria issued by ICTV, one virus isolate will be classified as the same species if the CP nucleotides have >76% homology (Wylie et al., 2017). Farida et al. (2022) stated that the PRSV isolate isolated from Java had high homology similarities to PRSV Thailand.

However, based on CP phylogenetic analysis, the Sukaraja isolate was separated from the Javanese isolate and even closer to the Malaysian and Oklahoma isolates. In this study, the identification of the CP gene is still

partial (Figure 4). So, further studies are still needed in the form of complete sequence analysis of CP isolates for more accurate species determination. It is also known that PRSV transmission has occurred since 2012 and has spread from Aceh, North Sumatra, Java, and Bali (Hidayat et al., 2012). Therefore, the chronology of the origin of these two PRSV isolates and their relationship with PRSV from Thailand, Malaysia, and Oklahoma cannot be ascertained.

Conclusion

The symptoms of ring spot diseases found in papaya plantations show differences between Sukaraja and Cijeruk locations, but both typical symptoms revealed PRSV attacks. Inoculation of Sukaraja and Cijeruk PRSV isolates on five types of plants showed symptoms that were different from those obtained in the field and also differed between plants. Both isolates showed the symptomps on papaya, bitter melon, and melon, but did not produce symptoms on eggplant and chickpeas. The two isolates had a homology percentage of 97.7% with the PRSV isolate from Thailand and 93.07-99.8% with the Kulon Progo and Nganjuk isolates. The Cijeruk isolate was the same branch as the PRSV isolate from Indonesia, while the Sukaraja isolate had a separate branch and was related to isolates from Oklahoma and Malaysia. The results contribute new information regarding the nucleotide diversity of PRSV CP region sequences.

Declaration

Author contribution

Riska is the main contributor and corresponding author for this paper. Eko Darma Husada, Tri Budiyanti and Jumjunidang are co-authors. All authors read and approved the final paper.

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Competing interest

The authors declare no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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