



## Research Article

# The Existence of *Papaya ringspot virus*-Papaya Strain on Cucumber in Gianyar, Bali

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## ABSTRACT

Yellow mosaic symptoms were identified from cucumber production systems in Gianyar and were similar to symptoms of *Papaya ringspot virus* infection. Further research was conducted to determine diseases incidence and molecular characteristic of *Papaya ringspot virus*. Ninety leaf samples were collected from Gianyar by purposive sampling and disease incidence calculations were based on symptoms in the field. Detection and identification were done using a RT-PCR with specific primers of CP PRSV-P, CP PRSV-W and DNA sequencing. Disease incidences in the fields ranged between 5.81–66.87%. Specific DNA band 470 bp was successfully amplified from several cucumber leaf samples collected from Ubud, Payangan, Tegallalang, Sukawati, Gianyar, and Blahbatuh; but no DNA were amplified from all samples when using CP PRSV-W specific primer. Nucleotide and amino acid analysis showed nucleotides homology to isolates from Ubud, Payangan, Tegallalang, Sukawati, Gianyar, and Blahbatuh, i.e. 98.9–99.5% and 99.1–100%, respectively. Results indicated that genetic variation of PRSV-P from Gianyar was low. Furthermore, the nucleotides homology of PRSV-P isolates from Ubud, Payangan, Tegallalang, Sukawati, Gianyar, and Blahbatuh were with PRSV-P isolates which infected cucumbers from Nganjuk (LC311783) and Brebes (LC311784), while from native papaya collected in Bali Bali (LC223115) were 97.2–98.4% and 98.6–100%, respectively. Phylogenetic analysis confirmed that PRSV-P isolates from Indonesia were in the same cluster with Thailand isolates. The results showed that sources of PRSV-P inoculums spreading into new areas.

Keywords: nucleotide sequences; phylogenetic analysis; PRSV-P

## INTRODUCTION

*Papaya ringspot virus* (PRSV) is a member of Potyvirus genus, which is known to be widely distributed. PRSV can be transmitted by aphid vectors, such as *Aphis gossypii*, *A. craccivora*, and *Myzus persicae*, non-persistently, mechanically and not seed-borne (Kalleswaryaswamy & Kumar, 2008). PRSV causes mosaic symptoms on leaves, stems and stalks, as well as dark green spots on papaya fruit (Hidayat *et al.*, 2012). In early 2012, PRSV-P strain was first reported to infect papaya plants in Aceh-Indonesia. Afterwards, this disease was also reported in Medan, Bogor, and Bali with disease incidence up to 100% (Hidayat *et al.*, 2012; Harmiyati *et al.*, 2015; Nurhantoro *et al.*, 2018).

Based on different host plants, PRSV is divided into PRSV-P and PRSV-W strains (Gonsalves *et al.*, 2010) where PRSV-P infects Caricaceae and Cucurbitaceae, while PRSV-W only infects Cucurbitaceae. PRSV-P infection was first reported in 1949 in Hawaii and the same strain was also reported to infect papaya plants in Polynesia and North Africa in 2005 (Tennant *et al.*, 2007). The PRSV-W strain infection was first reported from Australia in 1991, then followed by Sudan and Cuba in 2015 (Gonsalves *et al.*, 2010; Mohammed *et al.*, 2012; Martinez *et al.*, 2015; Mansilla *et al.*, 2013). PRSV-P infection has also been reported in China on *Momordica charantia* (Zhu *et al.*, 2016). This the potential of Cucurbitaceae species as a host PRSV-P.

In Indonesia, the PRSV-P strain was first reported by Harmiyati *et al.* (2015). This research showed that PRSV-P isolates from Medan and Aceh caused mild mosaic symptoms on Cucurbitaceae with disease severity of 40% compared to 100% on Caricaceae. The PRSV-P molecular characters of Javanese isolates have been reported by Listihani *et al.* (2018).

The results of a disease survey on cucumber plant from Gianyar, Bali which was carried out in 2020, found yellow mosaic symptoms similar to ones caused by PRSV-P (Laili & Damayanti, 2019). Therefore, this study was conducted to determine disease incidence and to further identify viruses molecularly.

## MATERIALS AND METHODS

### Survey and Sampling

Samples were collected from cucumber production system in Gianyar Regency (Tampaksiring, Ubud, Payangan, Tegallalang, Sukawati, Gianyar, and

Blahbatuh sub-districts) (Figure 1). Sampling was carried out by purposive sampling method; 10 symptomatic samples were taken from each location. The total samples taken were 90 and used as material for virus detection. Based on symptoms of viral infection in the field, the incidence of disease (ID) was calculated as:

$$ID = \frac{\text{The number of samples symptomatic}}{\text{Total samples in the field}} \times 100\%$$

### RNA Extraction

Total RNA was extracted from symptomatic plant leaf tissues using the CTAB method. Total viral RNA was isolated from infected leaf following a procedure described by Doyle and Doyle (1987). Fresh tissue (0.1 g) was grinded with liquid nitrogen and 500  $\mu$ L of 10% CTAB buffer (cetyl-trimethylammonium bromide, 0.1 M Tris-HCl pH 8, 0.05 M EDTA, 0.5 M NaCl, 1%  $\beta$ -mercaptoethanol) was added.

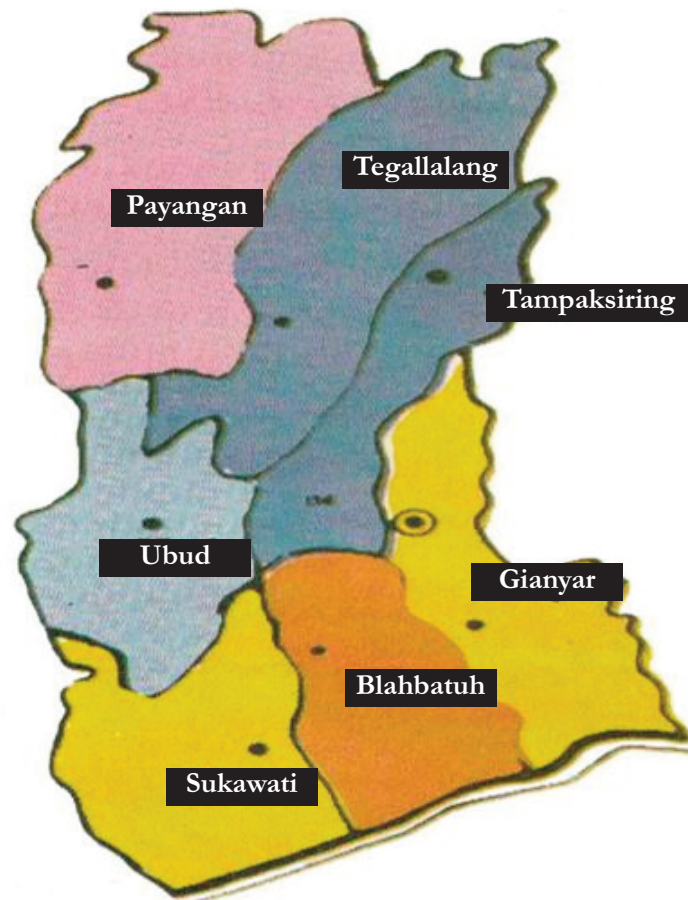


Figure 1. Sampling location of cucumber leaves showed symptoms caused by PRSV-P in Gianyar, Bali (Scale1 : 350,000).

Results were then transferred to 1.5 ml micro tubes and incubated in a water bath at 65°C for 1 hour (30 minutes for total RNA extraction), then the micro tubes every 10 minutes to separate the lipids and proteins. After incubated in a water bath, the micro tubes containing the mixture was taken from the water bath and allowed to stand for 2 minutes at room temperature, then 500 µL of chloroform: isoamylalcohol mixture with a ratio of 24: 1 (v/v) was added. The mixture was homogenized for 5 minutes, then centrifuged at 14,000 rpm for 15 minutes. A total of 450 µL of supernatant was taken and transferred into a new micro tube, then added 3 M of sodium acetate 1/10 of the volume of the supernatant (45 µL) and 2/3 of the volume of the supernatant (300 µL), then mixed in a tube. The mixture was homogenized and incubated at -80°C for 2 hours or -20°C overnight. After incubation, the nucleic acid mixture was centrifuged at 12,000 rpm for 10 minutes to precipitate the nucleic acids. The nucleic acid pellets were washed by adding 500 µL of 70% ethanol, then centrifuged again at 8,000 rpm for 5 minutes, then dried. After drying, pellets containing total RNA were dissolved in 50 to 100 µL of nucleic free water or TE buffer (pH 8) and the nucleic acid was stored at -20°C until ready for further processing.

### cDNA Synthesis

Total RNA extraction product was used as a template for cDNA synthesis. The composition of reverse transcription (RT) consisted of 1 µl oligo d (T) 10 mM, 2 µl total RNA, and 3.75 µl dH<sub>2</sub>O. All the reagents were evenly mixed and incubated at 65°C for 5 minutes, then immediately cooled in ice. The reactants consisting of 2 µl of RT buffer, 1 µl dNTP 10 mM, 0.5 µl DTT 50 mM, 0.5 µl RNase inhibitor (RiboLock RNase Inhibitor 20 units/µl, Thermo scientific), 0.5 µl MmuLV (Revertaid 200 units/µl, Thermo Scientific) to a total volume of 10 µl were added. The reverse transcription reaction was carried out at 42°C for 60 minutes followed by 70°C for 10 minutes to inactivate the enzymes. The cDNA product was then used as a template at the amplification stage.

### Amplification of RNA by RT-PCR

The primary pair used to amplify PRSV-P and PRSV-W were PRSV326 (5'-TCGTGCCACTCAAT CACAAT-3')/PRSV800 (5'-GTTACTGACACTG CCGTCCA-3') with a target amplicon size of 475 bp,

and PRSV-W 9016F (5'-CTTGARCARGCT CCA TTC-3')/PRSV-W 10253R (5'-CTAAAAGCA CG GAGG-3') with a target amplicon size of 1,238 pb (Mohammed *et al.*, 2012; Martinez *et al.*, 2014). As much as 25 µl amplification reaction consisted of 12.5 µl Go Taq green 2x (Thermo scientific), 1 µl 10 µM reverse primer, 1 µl 10 µM forward primer, 9.5 µl nuclease-free water, and 1 µl cDNA.

### DNA Visualization

DNA was electrophoresed on 1% Agarose in 0.5x TBE buffer. Samples were added with fluoroVuo nucleic acid dye (Smobio, Taiwan) and then electrophoresis at 100 V for 20 minutes. DNA was visualized under an ultraviolet transilluminator and documented with digital camera.

### DNA Analysis

The DNA fragments that have been PCR amplified were sent to First Base Malaysia for the nucleotide tracing process. Nucleotide were traced to determine the similarity level compared to nucleotide sequences stored in GenBank using the Basic Local Alignment Search Tool (BLAST) program. Matrix homology identities were analyzed using BioEdit software, and phylogenetic trees were analyzed using MEGA v6.0 software using bootstrap 1000 times.

## RESULTS AND DISCUSSION

### Disease Symptoms in the Field

Infected plants were easily recognized in the field due to their unique symptoms, which included appearance of mosaic, vein cleansing, malformations, and yellowing (Table 1). These symptoms are very common in viral infections and are only sufficient for the initial diagnosis of viral infection due to symptoms variation that may occur by different cultivars, plant age, and environmental conditions (Harmiyati *et al.*, 2015). Symptoms variation of PRSV on papaya plant has also been reported by Hidayat *et al.* (2012). The common symptoms on cucumber plant showed yellow mosaic (Figure 2A) and malformations (Figure 2B). The incidence of disease in the field ranged from 5.81–66.87% (Table 1).

### Detection of PRSV using RT-PCR

Specific DNA fragment of 470 bp was successfully amplified from several field samples that showed yellow mosaic symptoms (Figure 3). In contrast,

Table 1. Disease symptoms and incidence of PRSV-P on cucumber in Gianyar

Location	Plant age (days)	Disease symptoms	Disease incidence (%)
Payangan	33–40	Yellow mosaic, yellowing, mottle, and malformation	33.97 (123/362)
Tegallalang	24–42	Yellow mosaic, vein banding, yellowing, and malformation	37.83 (213/563)
Tampaksiring	28–37	Vein banding, yellowing, and malformation	19.11 (134/701)
Ubud	35–42	Yellow mosaic, yellowing, vein banding, and mottle	66.87 (523/782)
Sukawati	20–33	Yellow mosaic dan yellowing	5.81 (42/739)
Blahbatuh	30–43	Yellow mosaic, vein clearing, vein banding, and mottle	63.80 (520/815)
Gianyar	33–46	Yellow mosaic, yellowing, vein banding, vein clearing, and malformation	31.67 (134/428)

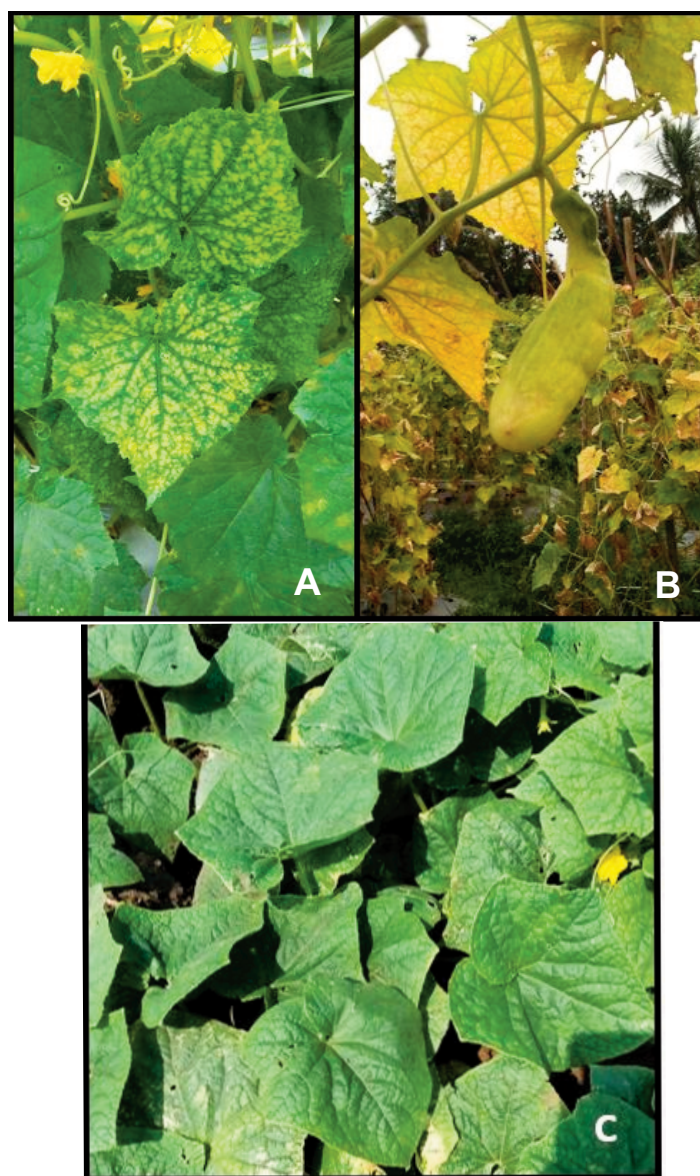


Figure 2. Disease symptoms on cucumber plants in Gianyar: (A) yellow mosaic on leaves, (B) fruit malformation, and a healthy plant (C)

no DNA bands were obtained from all samples that used PRSV-W primer (data not shown). The same primer was used by Listihani *et al.* (2018) and was successfully to amplified PRSV-P from cucumber plants collected in Java. Harmiyati *et al.* (2015) detected PRSV-P infection from papaya plants in Bogor using PRSV326/PRSV800 primers.

### Identification and Molecular Characterization of PRSV

Nucleotide and amino acid sequences showed homology, 91.8–98.4% and 95.7–100%, respectively, with the PRSV sequences contained in GenBank database (Table 1). The homology of PRSV-P nucleotides and amino acids between Gianyar isolates 98.9–99.5% and 99.1–100%, respectively. PRSV-P sequences from Ubud, Payangan, Tegallalang, Sukawati, Gianyar, and Blahbatuh showed similar homology with isolates of cucumber plants collected from Nganjuk (LC311783) and Brebes (LC311784), and Bali-Indonesia isolate (LC223115) on papaya 97.2–98.4% and 98.6–100%; while the homology with PRSV-W from Brazil (DQ374152) was 89.4–90.9% (%). Harmiyati *et al.* (2015) also reported that PRSV-P among Indonesian isolates have similar homology compared to isolates from other countries. The homology of the PRSV coat protein gene among isolates indicated less diversity among PRSV-P isolates. Phylogenetic analysis showed that PRSV-P were clustered into 4 groups, namely groups I, II,

III, and IV, and were separated from PRSV-W and WMV as an out group. The PRSV-P isolates that were collected from Ubud, Payangan, Tegallalang, Sukawati, Gianyar, and Blahbatuh, were clustered into 1 group with isolates from Indonesia and Thailand (Figure 4). PRSV-P, which infect cucumber plants, suspected to be transmitted by vectors from papaya. In addition, it also possible that cucumber plants have already been infected with PRSV-P, but has not been reported.

Previous studies reported that PRSV-P able to infect cucumber plants through physical inoculation (Singh *et al.*, 2017). Furthermore, there has been first reported of infection and molecular characterization of PRSV-P in cucumber plants from Java (Listihani *et al.*, 2018). The presence of PRSV-P in cucumber plants in Gianyar, Bali source of inoculum within the field.

The Regulation of the Minister of Agriculture Number 51/Permentan/Kr.010/9/2015, regarding Types of Quarantined Plant Destruction Organisms, states PRSV as a quarantine plant pests (OPTK), category A2. Report from Philippines this virus can be transmitted from seeds at a very low rate of 0.15% (Bayot *et al.*, 1990). PRSV-P able to infect papaya or cucumber, although the latter is less likely because natural transmission from papaya to cucumber does not happen (Bateson *et al.*, 2002). PRSV-P closely related to the strain of watermelon virus (PRSV-W), which causes important diseases

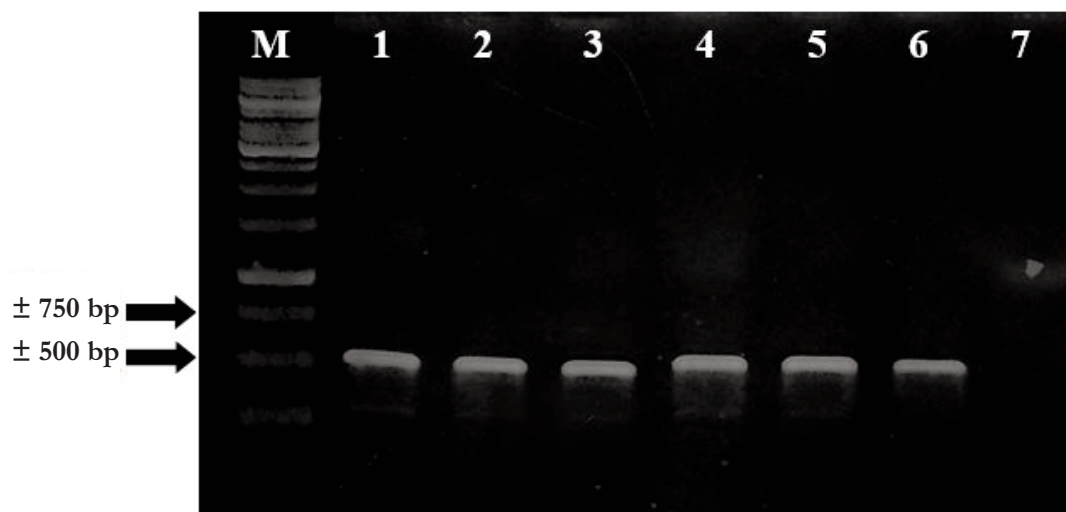


Figure 3. DNA visualization of PRSV-P using primer pairs of PRSV326/PRSV800. Samples from: Payangan (1), Tegallalang (2), Ubud (3), Blahbatuh (4), Sukawati (5), Gianyar (6), and Tampaksiring sub-districts (7); M. 1 kb DNA marker (Thermo Scientific)

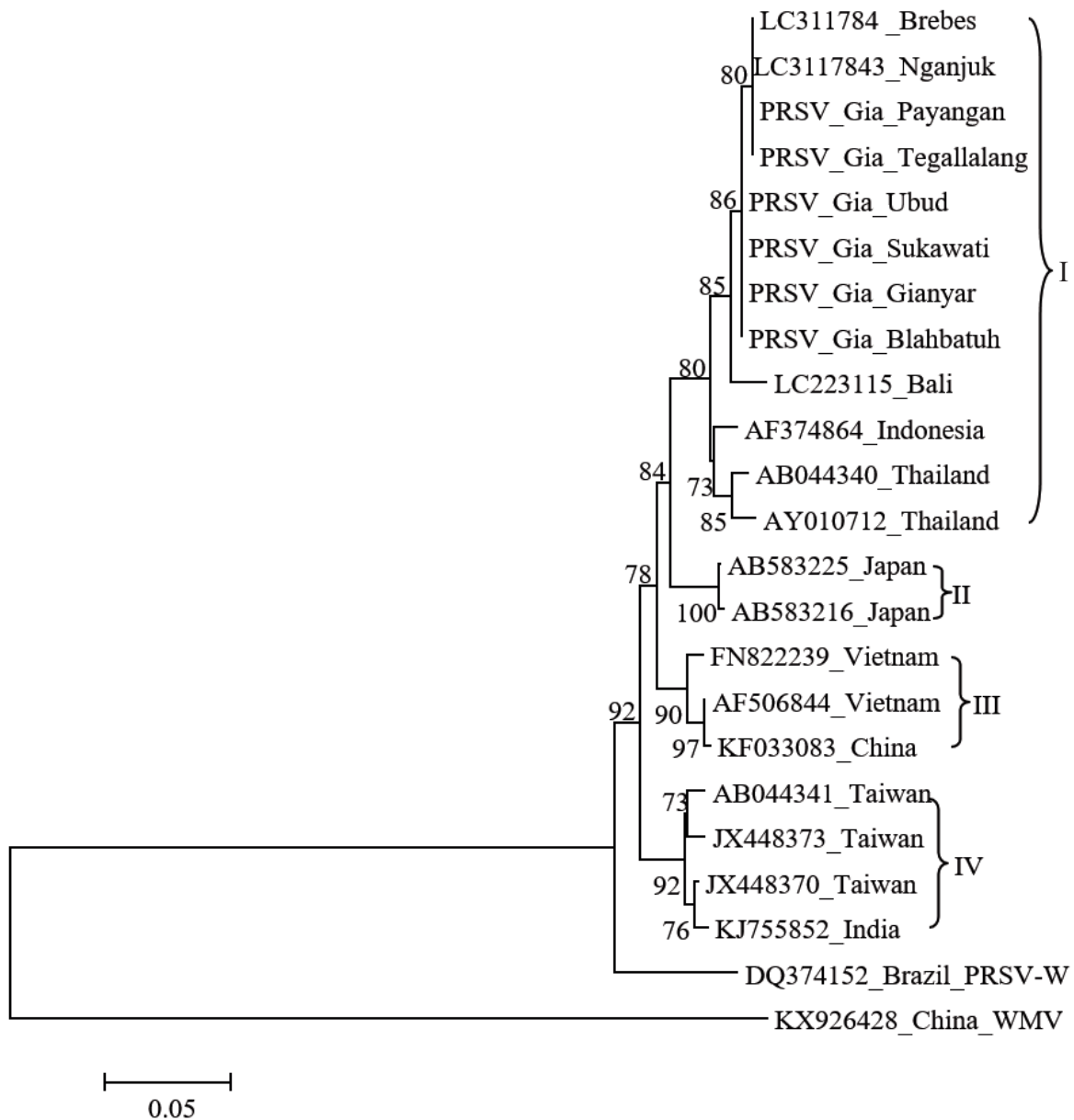


Figure 4. Phylogenetic tree of PRSV-P which infect on cucumber cultivations in Gianyar using software MEGA 6.0 with *Neighbour Joining* to 1000x bootstrap.

on cucumber plants globally (Davis *et al.*, 2005). Analysis of the PRSV coat protein gene sequence that elsewhere in the world PRSV-P has mutated into PRSV-W (Bateson *et al.*, 2002). In Queensland, Australia, it took more than 20 years for PRSV-P to appear after the first detection of PRSV-W (Thomas & Dodman, 1993). Papaya production with cucumber intercropping in recent years caused by PRSV P that infected cucumbers in Gianyar, Bali.

## CONCLUSION

PRSV-P was successfully identified from cucumber cultivation in Gianyar, Bali with nucleotide homology similarities with Nganjuk and Brebes isolates. The PRSV-P has spread in Java and Bali, therefore this PRSV-P report on cucumber in Bali is the first report.

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