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Distribution and Identification of *Pepper yellow leaf curl Indonesia virus* Infecting Chili Pepper in Bali

Distribusi dan Identifikasi *Pepper yellow leaf curl Indonesia virus* yang Menginfeksi Tanaman Cabai di Pulau Bali

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ABSTRACT

Yellow leaf curl disease in chili pepper has been reported in Bali Island since the early 2012. Research was conducted to identify the virus causing this disease and disease distribution in Bali. Field survey was carried out to observe disease intensity and to collect field samples from several chili pepper growing areas in Bali (Karangasem, Bangli, Tabanan, and Gianyar). Begomovirus identification from field samples was then conducted by polymerase chain reaction method using universal primers SPG1/SPG2, followed by an analysis of the amplified target DNA sequences. The incidence of pepper yellow leaf curl disease reached 100% at all sites and disease severity reached 18%−87%. Begomovirus specific DNA fragment measuring 912 bp was successfully amplified from 12 field samples. Sequence analysis of DNA fragments showed the highest homology with *Pepper yellow leaf curl Indonesia virus* (PYLCIV). Further phylogenetic analysis confirmed the relationship between PYLCIV isolates from Bali and various PYLCIV isolates from Indonesia.

Keywords: disease incidence, phylogenetic analysis, polymerase chain reaction, sequence analysis, universal primer

ABSTRAK

Gejala daun keriting kuning pada cabai telah dilaporkan di Pulau Bali sejak awal tahun 2012 dan saat ini semakin meluas. Penelitian dilakukan untuk mengidentifikasi penyebab penyakit dan daerah sebarnya di Pulau Bali. Penelitian diawali dengan pengamatan keparahan penyakit dan pengambilan sampel lapangan dari beberapa daerah penanaman cabai di Bali (Karangasem, Bangli, Tabanan, dan Gianyar). Identifikasi Begomovirus dari sampel lapangan dilakukan dengan metode *polymerase chain reaction* menggunakan primer universal SPG1/SPG2 dan dilanjutkan dengan analisis sikuen DNA target yang telah diamplifikasi. Insidensi penyakit daun keriting kuning cabai mencapai 100% pada semua lokasi pengamatan dan keparahan penyakit berkisar antara 18%−87%. Fragmen DNA spesifik Begomovirus berukuran 912 pb berhasil diamplifikasi dari 12 sampel lapangan. Analisis sikuen fragmen DNA menunjukkan homologi tertinggi dengan *Pepper yellow leaf curl Indonesia virus* (PYLCIV). Analisis filogenetik lebih lanjut mengonfirmasi hubungan kekerabatan antara isolat-isolat PYLCIV dari Bali dengan berbagai isolat PYLCIV dari Indonesia.

Kata kunci: analisis filogenetika, analisis sikuen, insidensi penyakit, primer universal, *polymerase chain reaction*

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INTRODUCTION

Yellow leaf curl disease is considered the most important disease in chili pepper in Indonesia due to its effect on yield loss. Setiawati *et al.* (2008) reported 20% to 100% yield loss due to yellow leaf curl disease in Central Java when it first occurred. The disease is now spreading fast across the country especially in the production region of chili pepper such as in Central Java, West Java, Yogyakarta, Lampung, Bali, and Nusa Penida Island (Sulandari *et al.* 2006; Selangga *et al.* 2019; Selangga and Listihani 2021).

The causal agent of yellow leaf curl disease in chili pepper in Java has been identified as *Pepper yellow leaf curl Indonesia virus* (PYLCIV), a member of Begomovirus (Sulandari *et al.* 2006). Recently, Annisaa *et al.* (2021) also confirmed PYLCIV as the main Begomovirus infecting chili pepper in Yogyakarta. Members of Begomovirus are known to have a high genetic diversity. Research on the molecular characters of Begomovirus has been reported by Santoso *et al.* (2008) showed genetic diversity among *Tomato leaf curl virus* (ToLCV) infecting tomatoes in Java and Sumatra. Lee *et al.* (2011) also reported recombinations between *Tomato yellow leaf curl virus* (TYLCV) Korea isolates and other TYLCV isolates, including those from Thailand (AF206674), Iran (AJ132711), and Israel (X76319). Molecular characterization of PYLCIV indicated some sequence variation among different isolates, although further studies are required to confirm whether this variation leads to genetic diversity.

The symptom of yellow leaf curl disease in chili pepper was first observed in Bali in 2012. At that time, disease incidence reached 22.75% in Kertha, Payangan, Gianyar (Putra *et al.* 2015). A significant yield loss in chili pepper production was reported that might be caused among others by this disease (BPS Bali 2015). Therefore, a field survey needs to be done to determine disease intensity due to the increasing of chili growing areas in Bali, especially in several regencies such as Bangli, Tabanan, Karangasem, and Gianyar. This research aims to identify the virus causing yellow leaf curl disease of chili pepper and to determine the distribution of this disease in Bali.

MATERIALS AND METHODS

Field Survey and Samples Collection

Survey was conducted in July 2017 in several chili pepper growing areas in Karangasem, Tabanan, Bangli, and Gianyar Regencies. Observations were made on the specific symptoms involving yellow mosaic, leaf curl, green mosaic, and dwarfing. Sampling at each location were conducted using diagonal method, in which 50 plants were taken as sample plants. Disease incidence (DI) was measured as the proportion of diseased plants in a population, regardless of the weight or severity, to the total of plant samples; while disease severity (DS) was assessed based on disease score as described by Ganefianti *et al.* (2008) (Table 1), following the formula below:

$$DS = \frac{\sum_{i=0}^{i}(n_i \times v_i)}{Z \times V} \times 100\%, \text{ dengan}$$

with n_i, number. infected plants in the same category; v_i, score of severity; Z, maximum rating score; N, total number. plants observed.

Leaf samples showing symptoms were taken from each location for further identification in the laboratory.

Detection and Identification of Begomovirus

Leaf samples were subjected for Begomovirus detection by polymerase chain reaction method using universal primers SPG 1 (5'-CCCCKGTGCGWRAATCCAT-3') and SPG 2 (5'ATCCVAAYWTYCAGGGAGCT-3') (Li *et al.* 2004). Total DNA isolation from leaf samples was performed using CTAB method (Doyle and Doyle 1987). Liquid nitrogen was added to preserved leaf tissue (0.1 g), the tissue was finely ground in a mortar, then transferred to 2.0 mL microtubes. Five hundred microliter of extracting buffer solution (20 mM EDTA, 100 mM Tris-HCl pH 8, 1.4 M NaCl, 2% CTAB, and 0.2% mercaptoethanol) was added on to the microtube followed by incubation at 65 °C for 60 minutes with occasional mixing by

Table 1 Disease score based on visual symptoms in plant

Score	Symptoms
0	Symptomless
1	Yellow leaves
2	Yellow and curly leaves
3	Yellow and cupping with leaf edges curl upward or downward
4	Yellow, curly and cupping of the leaves
5	Plant dwarfing with leaf yellowing and malformation

gently inverting the tube. After incubation, a volume of phenol: chloroform: isoamyl alcohol (25:24:1) was added and the tube was inverted several times followed by centrifugation at 12 000 rpm for 15 minutes. The upper phase was transferred to a new microtube followed by adding sodium acetate (1/10× volume) and cold isopropanol (2/3× volume), then the mixture was incubated overnight at -20 ℃ to precipitate DNA. After the incubation, the tube was centrifuged at 12 000 rpm for 10 minutes and the supernatant was removed. The pellet containing total DNA was washed with 70% ethanol and centrifuged at 8000 rpm for 5 minutes. Pellet DNA was air-dried and resuspended in TE buffer solution (1×) and stored at -80 ℃ for further use.

Amplification reaction was prepared using ready to go PCR bead (Thermo Scientific). For each reaction, 2 µL of DNA, 1 µM of each SPG1/SPG2 primer, and distilled water to final reaction volume of 25 µL was added to a PCR bead. DNA amplification was conducted in a thermal cycler (Gene Amp, PCR System 9700 PE Applied Bio-system) started with a pre-heating cycle for 5 minutes at 94 ºC, followed by 35 cycles of amplification consisted of denaturation (1 minute at 94 ℃), annealing (1 minute at 50 ℃), and extension (1 minute at 72 ℃). The last cycle ended at 72 ℃ for 1 minute and cooled down to 4 ℃ (Rojas *et al*. 1993). The amplicon was then visualized by electrophoresis using 1% agarose gel in 0.5× TBE (Tris-Boric acid-EDTA) buffer. The electrophoresis was performed at 50 V for 50 minutes, then the gel was soaked on to 0.1% EtBr for 10 minutes, washed with H_2O for 20–30 minutes, and visualized under UV transilluminator.

Sequence Analysis

PCR product was subjected to direct sequencing and the sequence data was analyzed using BioEdit V.7.0.5 software program, CLC Sequence Viewer 8, and MEGA 6.06.

RESULTS

Disease Incidence and Severity

Incidence of yellow leaf curl disease in all locations reached 100% with disease severity ranged from 18% to 87% (Table 2). Infected plants were easily recognized in the field due to their unique symptoms, involving yellow and green mosaic, mottle, leaf curl, leaf cupping upward and/or downward, and plant dwarfing (Fig 1). This type of symptoms was known as typical symptoms for yellow leaf curl disease of pepper as it was also reported occurred in Sumatera and Java (Sudiono *et al.* 2005; Annisaa *et al.* 2021).

Identification of PYLCIV

Detection of samples by PCR using universal primers confirmed the infection of Begomovirus. The expected DNA fragment of 912 bp was successfully amplified from all field samples (Fig 2). Samples representing survey locations in Bali were selected for further identification by direct sequencing. The partial sequences of the Begomovirus genome obtained from these DNA fragment contains partial replication genes (AC2) and transcriptional activator (AC1) (Li *et al.* 2004). Nucleotide sequences of each isolates collected from this study has been submitted to GenBank with the accession number as listed in Table 3.

Sequences analysis from all isolates showed their highest homology (>94%) with other isolates of PYLCIV from Indonesia; but their sequence homology are low (65% to 70%) to PYLCV from Thailand (KX943290) and India (JN663870) (Table 3). Phylogenetic analysis indicated that isolates from Bali could be differentiated into three groups, whereas the

Table 2 Field symptoms and severity of pepper yellow leaf curl disease in Bali

Location (Village, regency)	Field symptoms*	Disease severity (%)
Talibeng, Karangasem	Ym, m, gm, lc, cu, cd, d	84
Telaga Tawang, Karangasem	Ym, m, gm, lc, cu, cd, d	53
Pempatan, Karangasem	Ym, m, gm, lc, cu, cd	55
Perean Tengah, Tabanan	Ym, m, gm, lc, cu, cd, d	61
Bangli A, Tabanan	Ym, gm, lc, cd, d	48
Bangli B, Tabanan	Ym, gm, lc, cu, cd, d	60
Blanga, Bangli	Ym, m, gm, lc, cu, cd, d	87
Selulung, Bangli	Ym, gm, lc, cu, cd, d	18
Bayung Gede, Bangli	Ym, m, gm, lc, cu, cd, d	21
Kertha A, Gianyar	Ym, m, gm, lc, cu, cd, d	60
Kertha B, Gianyar	Ym, m, gm, lc, cu, cd	70
Ketewel, Gianyar	Ym, gm, lc, cu, cd, d	35

*Yellow mosaic (ym), mottle (m), leaf curl (lc), green mosaic (gm), dwarf (d), cupping upward (cu), and cupping downward (cd). Infection of Begomovirus was confirmed by PCR for all field samples showing symptom.

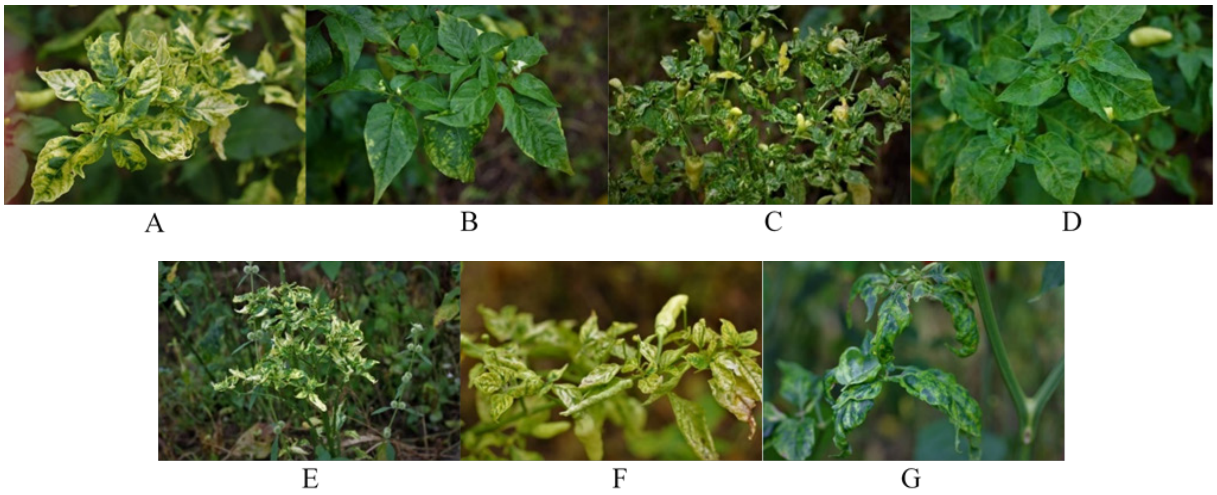

Figure 1 Symptoms of yellow leaf curl disease in chili pepper in Bali. A, Yellow mosaic; B, Mottle; C, Leaf curl; D, Green mosaic; E, Dwarfing; F, Cupping upward; and G, Cupping downward.

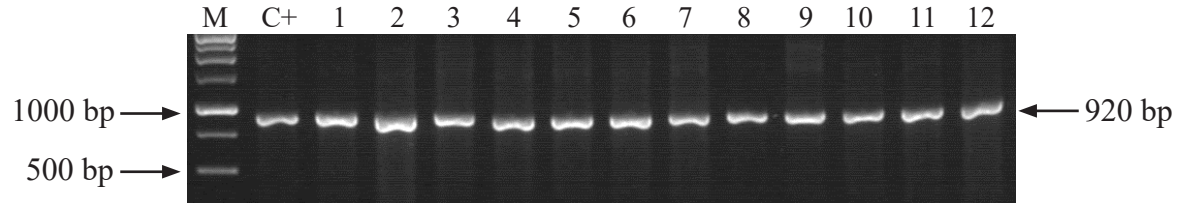

Figure 2 Visualization of Begomovirus specific DNA fragments amplified from leaf samples using universal primers SPG1/SPG2 on 1% agarose gel. DNA marker was 1 kb (M), PYLCIV-Brebes (AB267834) as positive control (C+), and field samples from Talibeng, Telaga Tawang, Pempatan, Perean Tengah, Bangli A, Bangli B, Blanga, Selulung, Bayung Gede, Kertha A, Kertha B, and Ketewel (column 1–12).

isolates of PYLCV from India and Thailand formed a separate group (Fig 3). Most of the isolates from Bali (TTwg 1, Tlbg 1, Ktwl 1, TTwg 2, Krth A1, Blng 2, Slng 1, Krth A2, Krth B1, BGde 1, Pmtn 1, and Krth B2) were clustered in the first group; 5 isolates (Bngl B1, PTng 1, Tlbg 2, Blng 1, and Slng 2) were in the second group together with PYLCIV Sumatera-Smt (LC051113), PYLCIV Bogor-Bgr (DQ083765), PYLCIV Java (JX416180), and PYLCV Bogor-Bgr (AB267838); and the other 2 isolates (PTng 2 and Bngl A1) were clustered in the third group.

DISCUSSION

Early infection of Begomovirus is commonly recognized by the appearance of a yellowish-green mosaic symptom on young leaves, as the infection progresses, leaves become yellowing, and curling and plants become stunted. This symptoms are easily recognized in the field especially when the incidence is very high as it is found in Karangasem, Tabanan, Bangli, and Gianyar Regencies. Several factors may contribute to disease incidence, among others are varieties of chili pepper grown, cropping patterns, and crop management applied. Most of the farmers in Bali grow the same chili varieties, i.e. local cultivar "Seret" and hybrid cultivar "Pelita 8" which are known susceptible to PYLCIV (Selangga *et al.* 2019). Two cropping patterns, monoculture and intercropping, are commonly found in the chili pepper growing areas in Bali. The disease was found to be more severe in the monoculture cropping pattern compared to the intercropping. According to Soetiarso and Setiawati (2010), intercropping patterns can reduce disease intensity, especially when plants from different families are planted. The selection of inter-cropping plants with chili pepper needs to

Table 3 Nucleotide sequences homology (%) of Begomovirus infecting chili pepper in Bali with PYLCV reported earlier in GenBank

Begomovirus infecting chili pepper in Bali		PYLCV isolates in GeneBank*					
Accession Number	Isolate	1	2	3	4	5	6
LC381258	Tlbg 1	93.3	94.3	94.1	93.3	69.0	65.4
LC381259	Tlbg 2	93.8	97.6	94.8	93.0	69.5	65.2
LC381260	TTwg 1	93.3	94.3	94.1	93.3	69.0	65.4
LC381261	TTwg 2	93.3	94.3	94.1	93.3	69.0	65.4
LC381262	PTng 1	94.8	98.2	95.9	94.1	69.8	65.4
LC381263	PTng 2	93.0	94.1	94.1	93.6	68.7	65.2
LC381264	Bngl A1	93.3	94.1	94.3	93.8	68.7	65.2
LC381266	Bngl B1	94.3	94.3	94.1	96.9	69.0	64.1
LC381268	Blng 1	94.1	97.9	95.1	93.3	69.5	65.2
LC381269	Blng 2	92.5	93.3	93.3	92.3	68.7	65.2
LC381270	Slng 1	93.0	93.8	93.8	92.8	69.0	65.4
LC381271	Slng 2	94.1	97.9	95.1	93.3	69.5	65.2
LC381272	Pmtn 1	91.8	93.0	92.5	92.5	68.5	65.7
LC381274	Krth A1	92.8	93.6	93.6	92.5	69.0	65.4
LC381275	Krth A2	92.8	93.6	93.6	92.5	69.0	65.4
LC381276	Krth B1	92.1	94.1	93.3	92.6	68.9	65.1
LC381277	Krth B2	92.1	92.8	92.3	92.3	68.2	65.4
LC381279	BGde 2	92.8	93.8	93.6	92.8	69.0	65.4
LC381280	Ktwl 1	93.3	94.3	94.1	93.3	69.0	65.4

*PYLCV in GeneBank: (1) *Pepper yellow leaf curl Indonesia virus* isolate Bogor-tomato (DQ083765_IDN_Bgr), (2) *Pepper yellow leaf curl Indonesia virus*-[Ageratum] (AB267838_IDN_Bgr), (3) *Pepper yellow leaf curl Indonesia virus* A-[Indonesia:Java] (JX416180_IDN_Java), (4) *Pepper yellow leaf curl Indonesia virus* isolate Sumatra (LC051113_IDN_Smt), (5) *Pepper leaf curl virus* isolate Pelampur, India (JN663870_PLCV_IND), and (6) *Pepper yellow leaf curl* Thailand virus isolate WF-SPN-Pep2015 (KX943290_PYLCV_THA).

consider the host range of Begomovirus to avoid other sources of disease inoculum in the field. Infection of PYLCIV has been reported on different plants, including tomato, chili, eggplant, and cucumber (Sakata *et al.* 2008; Annisaa *et al.* 2021). PYLCIV infection has also been reported on several weed species, including *Ageratum* spp. and Ludwigia (Sakata *et al.* 2008; Annisaa *et al.* 2021). During the survey, we observed some chili pepper fields with poor crop management in Tabanan and Gianyar. The crops did not grow well, weeds grew uncontrolled, and the whitefly population was fairly high. The transmission and distribution of PYLCV is known to be largely determined by the activity of the whitefly, *Bemisia tabaci* as its vector insect (Ganefianti *et al.* 2008). The polyphagous of the whitefly allows the transmission of Begomovirus from one plant to another.

Genetic diversity of Begomoviruses has been reported earlier. This is very likely to happen due to the wide host range of Begomovirus and its transmission manner by polyphagous whitefly. However, sequence analysis showed that PYLCIV isolates from

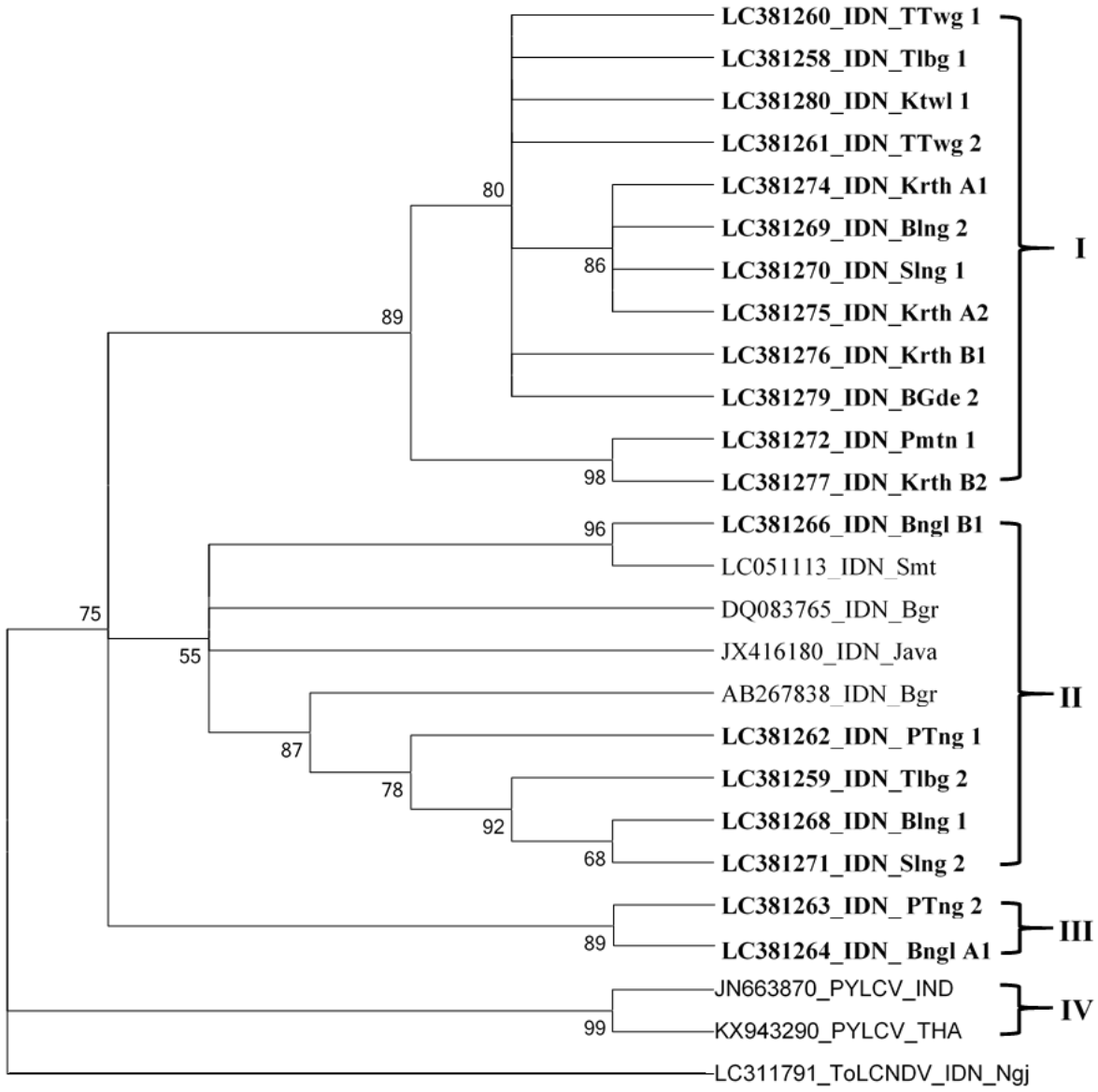

Figure 3 Phylogenetic analysis of Begomovirus infecting chili pepper in Bali based on alignment of partial nucleotide sequences of the DNA-A using Mega 6.06 (Algorithm Neighbor Joining with 1000 bootstraps replicates). *Tomato leaf curl New Delhi virus* from Nganjuk (ToLCNDV_IDN_Ng) is included as outgroup.

Karangasem, Tabanan, Bangli, and Gianyar have high homology with several other isolates from Indonesia, and low homology to isolates from India and Thailand. This indicates that PYLCIV infecting chili pepper in various regions in Indonesia is an indigenous strain/isolate. This is an important information for developing a strategy to control pepper yellow leaf curl disease in Indonesia, especially when using resistant varieties as the main approach.

In conclusion, this research confirmed the wide distribution of pepper yellow leaf curl disease in Bali. Nucleotide sequence analysis showed that PYLCIV isolates from Bali have a closed relationship with PYLCIV previously reported infecting chili pepper in Java.

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