Molecular Characterization of Begomoviruses Associated with Yellow Leaf Curl Disease in Solanaceae and Cucurbitaceae Crops from Northern Sumatra, Indonesia

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Begomoviruses, transmitted by whiteflies (Bemisia tabaci), have emerged as serious constraints to the cultivation of a wide variety of vegetable crops worldwide. Leaf samples from Solanaceae (tomato, tobacco, and eggplant) and Cucurbitaceae (cucumber and squash) plants exhibiting typical begomoviral yellowing and/or curling symptoms were collected in Northern Sumatra, Aceh province, Indonesia. Rolling circle amplification was conducted using DNA isolated from cucumber, squash, eggplant, and tobacco, and the full-length sequences of the begomoviruses were evaluated. The following viruses were isolated: bipartite begomoviruses Tomato leaf curl New Delhi virus (ToLCNDV), Squash leaf curl China virus (SLCCNV), Tomato yellow leaf curl Kanchanaburi virus (TYLCKaV), and a monopartite begomovirus Ageratum yellow vein virus (AYVV). Begomovirus diagnosis was conducted by PCR using begomovirus species-specific primers for Pepper yellow leaf curl Indonesia virus (PepYLCIV), Pepper yellow leaf curl Aceh virus (PepYLCAV), ToLCNDV, SLCCNV, TYLCKaV, and AYVV, which are the predominant begomoviruses. The primary begomovirus species for each plant were as follows: PepYLCAV for tomato, AYVV for tobacco, TYLCKaV for eggplant, ToLCNDV for cucumber, and SLCCNV for squash. This study provides valuable information for breeding begomovirus-resistant cultivars as horticultural crops.

Key Words: diversity, geminivirus, horticultural crop, host plant, Southeast Asia.

Introduction

Indonesia comprises more than seventeen thousand islands, and the total land area is 1,904,569 km², which is approximately five times larger than the Japanese land area. The current population is > 250 million people, and it is the 4th most populous country in the world. According to statistics from the Food and Agriculture Organization of the United Nations (FAO STAT), Indonesia produced 2.359 million tons of fresh chili/sweet pepper (Capsicum spp.), 0.963 million tons of tomato (Solanum lycopersicum L.), 0.535 million tons of eggplant (Solanum melongena L.), 0.152 million tons of tobacco (Nicotiana tabacum L.), 0.425 million tons of cucumber (Cucumis sativus L.), and 0.567 million tons of squash (Cucurbita spp.) in 2017. Indonesia currently has an expansive domestic market for horticultural crops, and growth will continue along with economic development.

Begomoviruses are transmitted by whiteflies in the Bemisia tabaci species complex. These viruses have emerged as serious constraints to the cultivation of a wide variety of vegetable crops worldwide (Navas-Castillo et al., 2011). The genus Begomovirus encompasses 409 viral species (ICTV, 2018); most begomoviruses have bipartite genomes comprising two circular DNA components of about 2,800 nucleotides (DNAs A and B), whereas monopartite begomoviruses generally have only DNA-A like as a genome.
In some monopartite begomoviruses, alphasatellite and betasatellite, formerly known as DNA-α and DNA-β, are also present (Hanley-Bowdoin et al., 2013). Tomato yellow leaf curl virus (TYLCV), a monopartite begomovirus originating from the Eastern Mediterranean, is distributed around the world and is seriously impacting tomato production (Mabvakure et al., 2016). TYLCV is currently ranked 3rd among the most economically and scientifically important plant viruses worldwide (Scholthof et al., 2011). However, in Southeast and East Asia, a wide variety of distinct local begomovirus species have been identified from tomato plants, whereas TYLCV has only been detected in Japan, China, and Korea (Kenyon et al., 2014).

In our previous study, geminivirus diagnosis was made by PCR using DNA extracted from chili pepper leaf samples collected in the fields at five local farms located in the suburbs of Banda Aceh, Aceh province, Indonesia in 2012 (Koeda et al., 2016). Pepper yellow leaf curl disease (PepYLCD) was observed in more than 81% of plants from all of the fields studied, and symptoms reached 100% at four out of five fields. Most of the chili pepper plants were infected by a mixture of viruses such as Pepper yellow leaf curl Indonesia virus (PepYLCIV) with another bipartite begomovirus, Tomato yellow leaf curl Kanchanaburi virus (TYLCKaV) and/or a monopartite begomovirus Ageratum yellow vein virus (AYVV). More recently, we identified a new bipartite begomovirus species, Pepper yellow leaf curl Aceh virus (PepYLCAV), that had infected chili pepper, tomato, and tobacco from samples collected in 2017 (Kesumawati et al., 2019).

From our preliminary study conducted in Aceh province, yellow leaf curl disease was observed on many Solanaceae horticultural crops (chili pepper, tomato, tobacco, and eggplant) and Cucurbitaceae (cucumber and squash). However, the begovirus species infecting eggplant, cucumber, and squash have not yet been clarified. Although mixed infections of multiple begomovirus species are frequently observed in chili peppers (Koeda et al., 2016), mixed infections in other plant species have not yet been examined. In this study, molecular identification of the begomoviruses infecting horticultural crops was conducted, and the diagnosis was made by PCR using newly developed begomovirus species-specific primers.

Materials and Methods

Virus sources, cloning, and sequence determination

Leaf samples of tomato (S. lycopersicum), tobacco (N. tabacum), eggplant (S. melongena), cucumber (C. sativus), and squash (C. maxima) plants exhibiting yellowing and/or curling symptoms were collected at Aceh province, Indonesia from 2012 to 2017 (Table 1; Fig. 1). Genomic DNA was extracted from the leaves using Nucleon PhytoPure (GE Healthcare, IL, USA). One cucumber, one squash, two eggplant, and two tobacco samples were randomly selected for isolation of

| Plant species | Collection date | Place of sampling | Number of collected samples |
|---------------|----------------|-------------------|-----------------------------|
| Eggplant      | 2017 Feb       | Banda Aceh        | 2                           |
|               | 2017 Feb       | Lambeugak         | 3                           |
|               | 2017 Oct       | Saree             | 11                          |
| Tomato        | 2017 Feb       | Saree             | 14                          |
|               | 2017 Jul       | Lambeugak         | 29                          |
| Tobacco       | 2016 Jul       | Lambeugak         | 3                           |
|               | 2017 Jul       | Lambeugak         | 24                          |
| Cucumber      | 2012 Oct       | Lambeugak         | 4                           |
| Squash        | 2017 Jul       | Saree             | 5                           |

Fig. 1. Symptoms of yellow leaf curl disease. (a) Chili pepper (C. annuum), (b) tobacco (N. tabacum), (c) tomato (S. lycopersicum), (d) eggplant (S. melongena), (e) squash (C. maxima), and (f) cucumber (C. sativus). Arrowheads indicate symptoms observed in each plant species.
their viral-genome DNA. Full-length begomoviral genomes were amplified from the extracted DNA by rolling circle amplification (RCA) using a TempliPhi DNA amplification kit (GE Healthcare). An RCA reaction was performed according to Koeda et al. (2015). The concatemers produced in the reaction were monomerized by restriction digestion with BamHI, HindIII, or PstI. Digested products were resolved by electrophoresis using 1% agarose gel, and the bands that corresponded to 2.8 kbp were purified using a Gel DNA Recovery Kit (ZYMO Research, CA, USA). The 2.8 kbp monomers were cloned into the appropriate site of a pBlueScript II SK (+) vector (Agilent Technology, CA, USA). Monomeric full-length clones were purified using a FastGene™ Plasmid Mini Kit (Nippon Genetics, Tokyo, Japan). Presence of an alphasatellite and betasatellite was examined by PCR using universal primers (Briddon et al., 2002; Bull et al., 2003). The full-length sequences for betasatellites were amplified by specific primers (Beta full F, R) (Table 2). PCR was performed using EmeraldAmp PCR Master Mix (Takara Bio). The PCR reaction mixtures were initially denatured at 94°C for 2 min, followed by 35 or 40 cycles at 94°C for 30 s, annealing temperature for 30 s, as listed in Table 2, and 72°C for 1 min, terminating with 3 min of final extension at 72°C. The amplified PCR products were subjected to electrophoresis, using 1.0% (w/v) agarose gel.

**Results and Discussion**

Leaf samples of Solanaceae and Cucurbitaceae plants with typical begomoviral yellowing and/or curling symptoms were collected at Banda Aceh, Lambeugak, Laweung, and Saree in Northern Sumatra, Aceh province, Indonesia, between 2012 and 2017. Distinct yellowing and curling were observed in chili pepper and tobacco plants (Fig. 1a, b), whereas the symptoms observed in the tomato and eggplant were generally slight (Fig. 1c, d). In squash and cucumber, yellowing was observed in the leaves (Fig. 1e, f). The same symptoms were observed in all of the other plants cultivated in these fields.

In our previous studies, full-length sequences of begomoviruses were aligned using MUSCLE (Edgar, 2004). Pairwise sequence identity comparisons were performed using the Species Demarcation Tool v1.2 program (Muhire et al., 2014). Phylogenetic analyses were conducted on MEGA7 using the Neighbor-joining method (Kumar et al., 2016). The bootstrap consensus of the tree was inferred from 1000 replicates.

**Begomovirus species-specific diagnosis by PCR**

DNA-A of PepYLCIV, PepYLCAV, and TYLCKaV; DNA-A like of AYVV; and DNA-B of Tomato leaf curl New Delhi virus (ToLCNDV) and Squash leaf curl China virus (SLCCNV) were detected using species-specific primers (Table 2). PCR was performed using EmeraldAmp PCR Master Mix (Takara Bio). The PCR reaction mixtures were initially denatured at 94°C for 2 min, followed by 35 or 40 cycles at 94°C for 30 s, annealing temperature for 30 s, as listed in Table 2, and 72°C for 1 min, terminating with 3 min of final extension at 72°C. The amplified PCR products were subjected to electrophoresis, using 1.0% (w/v) agarose gel.

**Sequence analysis**

The obtained sequences were assembled using ATGC software (Genetyx, Tokyo, Japan) and analyzed by conducting sequence similarity searches using BLASTn.

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**Table 2. Begomovirus species-specific primers used in this study.**

| Virus species specificity | Primer name | Primer sequence (5′–3′) | Fragment size (bp) | Annealing temperature | Cycle number | Reference |
|--------------------------|-------------|-------------------------|--------------------|-----------------------|--------------|-----------|
| ToLCMYB                  | Beta full F | ACCACTACGCTACGCAACGACC | 1345               | 60                    | 35           | This study |
|                          | Beta full R | TTCTACCTCCACGGGTACAC   |                    |                       |              |           |
| PepYLCIV                 | Pep uni F   | GTGYWGTAYCTTCTGGGAAAYTKGA | 666               | 58                    | 35           | Kesumawati et al., 2019 |
|                          | Pl uni R    | ACGCCTAAACGATTGTGGGCG  |                    |                       |              |           |
| PepYLCAV                 | Pep uni F   | GTGYWGTAYCTTCTGGGAAAYTKGA | 466               | 58                    | 35           | Kesumawati et al., 2019 |
|                          | PA uni R    | GAACTTCAAGGGGTGGGTC    |                    |                       |              |           |
| TYLCKaV                  | TYLCKaV uni F | TACATAATCAAGCTCGCGATATTACA | 831               | 65                    | 35           | This study |
|                          | TYLCKaV uni R | CATAGCAATGTGATTGTAAGGGATAC |            |                       |              |           |
| AYVV                     | AYVV uni F  | GTGGTARTGGGCAATTCAT    | 449                | 63                    | 35           | This study |
|                          | AYVV uni R  | GATYTTCARAGTACATACWGGGAATTACT |           |                       |              |           |
| ToLCNDV                  | ToLCNDV Uni F | CKKTTTCCTTCCTTATTCATYCKAC | 353               | 50                    | 40           | This study |
|                          | ToLCNDV Uni R | GGCAATSGTCCCGTAATACKTC |                |                       |              |           |
| SLCCNV                   | SLCCNV Uni F | YACGRTCTTCTMTARTCGTTGC | 396                | 60                    | 35           | This study |
|                          | SLCCNV Uni R | AACGTTCCRGACATGTGGAAGWC |                |                       |              |           |

K = G or T; M = A or C; R = A or G; S = C or G; W = A or T; Y = C or T
begomovirus were isolated from chili pepper, tomato, and tobacco (Koeda et al., 2016, 2017, 2018; Kesumawati et al., 2019). In the present study, full-length sequences of the infecting begomoviruses affecting eggplant, cucumber, squash, and tobacco were isolated by an RCA method (Table 3). TYLCKaV sequences were isolated from two samples of eggplant and a single sample from tobacco, which showed high similarity (above 99%) with TYLCKaV-[BA_B6] (DNA-A: LC051116, DNA-B: LC177332) isolated from chili pepper showing PepYLCiD in 2012 at Aceh. The AYVV sequence isolated from a tobacco sample showed high similarity (above 99%) with AYVV-[BA_D1-2] (DNA-A like: LC051119) isolated from chili pepper. Furthermore, a betasatellite was isolated from the same tobacco sample and showed high similarity with a Tomato leaf curl Malaysia betasatellite (ToLCMYB) (LC511782) from a chili pepper that was isolated in our previous study (unpublished data). However, infection with an alphasatellite was not observed in the tobacco sample. These results suggested that TYLCKaV and AYVV are capable of infecting various species of Solanaceae crops.

ToLCNDV sequences were isolated from a cucumber sample. DNA-A exhibited the highest similarity with sequence (AB330079) isolated from cucumber in Thailand in 1996 and DNA-B with sequence (AB613826) isolated from cucumber in Indonesia in 2008. SLCCNV sequences were isolated from a squash sample, and DNA-A showed the highest similarity with sequence (KY652743) isolated from a squash in East Timor in 2015, and DNA-B with sequence (HM566113) isolated from melon in China in 2010. DNA-A and -B of ToLCNDV and SLCCNV showed 85% and 71% similarity, respectively, to each other, which indicates that genetically similar begomovirus species are also infective to Cucurbitaceae crops, even when genetically distant from the begomoviruses infecting the Solanaceae crops.

Phylogenetic analysis was conducted using full-length DNA-A sequences obtained in the present and previous studies (Koeda et al., 2016; Kesumawati et al., 2019) and with begomoviral sequences obtained in other Asian countries (Fig. 2). AYVV is distributed widely from Southeast to East Asia. AYVV isolated in our studies showed similarity with a Malaysian isolate that was genetically distant from other isolates from China, Taiwan, and Japan (Ishigaki island). Phylogenetic analysis implied that ToLCNDV and SLCCNV originated from India, and isolates from this study showed the highest similarity with those found in Southeast Asian countries such as Thailand and Malaysia. TYLCKaV showed the highest similarity with these Indonesian isolates, and the phylogenetic relationship suggested that these isolates invaded from Thailand, which is consistent with the report of Kenyon et al. (2014). TYLCKaV sequences from Vietnam and Cambodia formed a different clade from those found in Thailand, Laos, China (Yunnan province), and Indonesia, indicating movement toward differentiation in Southeast Asia.

Wilisiani et al. (2019) detected PepYLCiV, TYLCKaV, and ToLCNDV from Solanaceae and Cucurbitaceae crops with a loop-mediated isothermal amplification assay. In the present study conducted in Aceh province, Indonesia, in addition to the ones mentioned before, PepYLCiV, SLCCNV, and AYVV were the predominant begomoviruses for horticultural crops. Begomovirus species-specific primers were developed according to the sequences obtained in our studies and similar sequences registered in GenBank. As shown in the phylogenetic tree (Fig. 2), similarity was high between PepYLCiV, PepYLCaV, and TYLCKaV, and was also high between ToLCNDV and SLCCNV. For ToLCNDV and SLCCNV, primer pairs to specifically detect DNA-A of each begomovirus species were difficult to design because of their highly similar sequences. Thus, primer pairs to detect DNA-Bs were

| Begomovirus species | Isolate | DNA molecule and size (nt) | Accession no. | Host       | Identity (%) | Virus Accession no. |
|---------------------|---------|---------------------------|---------------|------------|--------------|---------------------|
| TYLCKaV             | [BAEg-1]| DNA A (2752)              | LC511771      | S. melongena | 99.7         | TYLCKaV LC051116    |
|                     |         | DNA B (2752)              | LC511777      |             | 98.8         |                     |
|                     | [BAEg-49]| DNA A (2752)             | LC511772      | S. melongena | 99.7         | TYLCKaV LC051116    |
|                     |         | DNA B (2752)              | LC511778      |             | 99.0         |                     |
|                     | [BATa-20]| DNA A (2752)             | LC511773      | N. tabacum  | 99.5         | TYLCKaV LC051116    |
|                     |         | DNA B (2752)              | LC511779      |             | 99.3         |                     |
| AYVV                | [BATa-9]| DNA A (2750)              | LC511774      | N. tabacum  | 99.4         | AYVV LC051119       |
| ToLCMYB             | [BATa-9]| betasatellite (1345)     | LC511783      | N. tabacum  | 94.9         | ToLCMYB LC511782    |
| ToLCNDV             | [BACu-20]| DNA A (2739)            | LC511775      | C. sativus  | 97.5         | ToLCNDV AB330079    |
|                     |         | DNA B (2680)              | LC511780      |             | 92.7         |                     |
| SLCCNV              | [BASq-17]| DNA A (2736)            | LC511776      | C. maxima   | 95.0         | SLCCNV KY652743     |
|                     |         | DNA B (2713)              | LC511781      |             | 87.6         |                     |

Table 3. Identities and sizes of begomoviruses isolated in this study.
designed. AYVV had rather low similarity, less than 75%, to other bipartite begomoviruses. DNA samples of tomato, eggplant, cucumber, and squash used for an RCA reaction to clone the full-length begomoviral sequences in this study, were used as templates for PCR to check the specificities of each primer pair (Fig. 3).

For PepYLClV, DNA extracted from agroinoculated pepper No. 218 in our previous study (Koeda et al., 2018) was used as template for PCR. All the primer pairs successfully detected specific begomovirus species (Fig. 3). Diagnosis of begomoviruses infecting tomato, tobacco, eggplant, cucumber, and squash was made by PCR using these begomovirus species-specific primers (Table 4). TYLCKaV was detected in tomato and tobacco; however, the infection rate was highest in eggplant indicating that it is the primary host for TYLCKaV. This result is consistent with other studies that have reported that most TYLCKaV have been isolated from eggplants in Southeast Asia (Green et al., 2003; Ha et al., 2008; Tang et al., 2014; Bagewadi and Naidu, 2016; Zhang et al., 2018).

Interestingly, PepYLClV, PepYLClV2, and AYVV were undetected in tomato, eggplant, cucumber, and squash used for an RCA reaction to clone the full-length begomoviral sequences in this study, were used as templates for PCR. All the primer pairs successfully detected specific begomovirus species (Fig. 3). Diagnosis of begomoviruses infecting tomato, tobacco, eggplant, cucumber, and squash was made by PCR using these begomovirus species-specific primers (Table 4). TYLCKaV was detected in tomato and tobacco; however, the infection rate was highest in eggplant indicating that it is the primary host for TYLCKaV. This result is consistent with other studies that have reported that most TYLCKaV have been isolated from eggplants in Southeast Asia (Green et al., 2003; Ha et al., 2008; Tang et al., 2014; Bagewadi and Naidu, 2016; Zhang et al., 2018).

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the Cucurbitaceae crops. These results strongly suggest that the infecting begomovirus species has differentiated from Solanaceae to Cucurbitaceae crops. PepYLCIV is reported to be a recombinant between PepYLCIV infecting Solanaceae, the Pumpkin yellow mosaic virus, and ToLCNDV infecting Cucurbitaceae (Kesumawati et al., 2019). Tobacco generally has a broad spectrum of infecting viruses, but mix-infection of Solanaceae-infecting and Cucurbitaceae-infecting begomoviruses was not detected in this study. Mix-infection of these begomoviruses may be detected from weeds in these fields. Therefore, further evaluation using larger numbers of samples or host-range investigation for PepYLCAV is required to clarify how PepYLCAV emerged.

In this study, isolation of the infecting begomovirus species and PCR-based diagnosis were conducted, and this clarified the predominant begomovirus species for each horticultural crop. Begomovirus species-specific primers developed in our study will be useful for further phytopathological studies in other places in Southeast Asian countries, including Indonesia, where the same begomovirus species are present. This study provides valuable information for developing begomovirus-resistant cultivars in the future. Screening and breeding of begomovirus-resistant cultivars for chili pepper, tomato, eggplant, cucumber, and melon are ongoing research projects in our team.

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**Author contribution statement**

E. Kesumawati and S. Koeda designed experiment, interpreted the results, and wrote the manuscript. S. Okabe conducted isolation of virus sequences and PCR. M. Khalil, G. Alfan, P. Bahagia, N. Pohan, and S. Zakaria conducted field surveys and sampling. All authors read and approved the final manuscript.

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**Table 4.** Diagnosis of infecting begomovirus species.

| Host       | Number of samples collected | Infected begomovirus species |
|------------|-----------------------------|------------------------------|
|            |                             | PepYLCIV | PepYLCAV | TYLCKaV | AYVV | ToLCNDV | SLCCNV |
| Eggplant   | 16                           | 0 (0%)   | 0 (0%)   | 11 (69%) | 0 (0%) | 0 (0%) | 0 (0%) |
| Tomato     | 43                           | 3 (7%)   | 30 (70%) | 1 (2%)   | 7 (16%) | 0 (0%) | 0 (0%) |
| Tobacco    | 27                           | 0 (0%)   | 7 (26%)  | 5 (19%)  | 20 (74%) | 0 (0%) | 0 (0%) |
| Cucumber   | 4                            | 0 (0%)   | 0 (0%)   | 0 (0%)   | 0 (0%) | 3 (75%) | 1 (25%) |
| Squash     | 5                            | 0 (0%)   | 0 (0%)   | 0 (0%)   | 0 (0%) | 0 (0%) | 4 (80%) |

**Fig. 3.** Detection of PepYLCIV, PepYLCAV, TYLCKaV, AYVV, ToLCNDV, and SLCCNV by PCR using begomovirus species-specific primers. DNA of original samples used for isolating the full-length sequences by RCA (Table 3) were used as templates for PCR; Tobacco [BATa-9] DNA for PepYLCAV and AYVV, Tobacco [BATa-20] DNA for TYLCKaV, Cucumber [BACu-20] DNA for ToLCNDV, Squash [BASq-17] DNA for SLCCNV. For detection of PepYLCIV, DNA extracted from No. 218 (C. annuum) agroinoculated with PepYLCIV in our previous study (Koeda et al., 2018) was used as a template for PCR.
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