

Research

Molecular Detection and Identification of Begomovirus Infecting Cucumber (*Cucumis sativus*) in Terengganu, Malaysia

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ABSTRACT

The genus *Begomovirus* from the family *Geminiviridae* is responsible for causing significant economic losses to many important horticultural crops, including cucumber (*Cucumis sativus* L.). Begomovirus infection during the early stages of plant growth can lead to complete yield loss. Hence, the identification of begomovirus species is important to design a precise resistant breeding strategy. This study aims to detect the presence of begomovirus in typical symptomatic cucumber leaves, identify the species of begomovirus present, and investigate the evolutionary relationships with other reported begomoviruses using phylogenetic analysis. Leaf samples from symptomatic cucumber plants were collected from the Green World Genetics (GWG) research station and a farm in Lembah Bidong, Rhu Tapai, Setiu, Terengganu. To detect the presence of begomovirus, PCR was carried out using universal primers targeting DNA-A, DNA-B, and betasatellite regions. DNA-A and betasatellite fragments were amplified, but not DNA-B. The amplified partial sequences of DNA-A were then analysed and compared with other begomovirus sequences in the GenBank database managed by the National Centre for Biotechnology Information (NCBI). The newly isolated DNA-A sequence from cucumber was 100% identical to the tomato leaf curl New Delhi virus (ToLCNDV). The phylogenetic tree was divided into two groups: group A, consisting of the newly isolated DNA-A sequence from cucumber, ToLCNDV, followed by squash leaf curl China virus (SLCCNV) and a monopartite begomovirus *Ageratum yellow vein virus* (AYVV); and group B, consisting of tomato yellow leaf curl Kancharaburi virus (TYLCKaV), pepper yellow leaf curl Indonesia virus (PepYLCIV), and pepper yellow leaf curl Aceh virus (PepYLCAV). For the first time in cucumber, betasatellite is reported in association with ToLCNDV, a bipartite old-world begomovirus. This study provides a basis for the selection and breeding of begomovirus-resistant cucumber varieties in the future.

Key words: Begomovirus, *Cucumis sativus*, DNA-A, phylogenetics, Terengganu, ToLCNDV

Article History

Accepted: 1 May 2024
First version online: 30 June 2024

Cite This Article:

Goundar, S.P., Ramani, S., Mohd Radzi, N., Abu Bakar, M.F., Nur Fatihah, H.N. 2024. Molecular detection and identification of begomovirus infecting cucumber (*Cucumis sativus*) in Terengganu, Malaysia. Malaysian Applied Biology, 53(2): 81-92. <https://doi.org/10.55230/mabjournal.v53i2.2798>

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INTRODUCTION

Cucumber (*Cucumis sativus* L.) belongs to the Cucurbitaceae family and is an economically important fruity vegetable (Jia & Wang, 2021). In the Cucurbitaceae family, cucumber is closely related to melon, watermelon, pumpkin, and squash. Worldwide, cucumber (including gherkins) production reached 93.53 million tonnes in 2021 with China, Turkey, and Russia as the biggest producers (Food & Agriculture Organisation of the United Nations, 2021). In Malaysia, cucumber is the third largest vegetable crop in terms of harvested area (4821.81 ha) and production (96,353.06 tonnes) with an average yield of 20 tonnes/ha, with Johor, Perak, and Kelantan as the main production states (Department of Agriculture Malaysia, 2021).

Cucumber growth is significantly impeded by yellow mosaic and leaf curl diseases, which are attributed to the genus *Begomovirus* of the family *Geminiviridae* (Sohrab *et al.*, 2017). Family *Geminiviridae* consists of 520 species, out of which 445 species are in the genus *Begomovirus* alone, infect dicots, phloem-limited, and transmitted primarily by

the whitefly (*Bemisia tabaci* Genn) vectors (Zerbini *et al.*, 2017; ICTV, 2021). Geminiviruses cause substantial damage to crops and pose a persistent threat to agricultural productivity worldwide. The taxonomic classification of the *Geminiviridae* family encompasses 14 distinct genera based on their host range, evolutionary relationships, insect vectors, and genomic structure. Out of this, 13 genera, namely *Becurtovirus*, *Capulavirus*, *Citlodavirus*, *Curtovirus*, *Eragrovirus*, *Grablovirus*, *Maldovirus*, *Mastrevirus*, *Mulcrilevirus*, *Opunvirus*, *Topilevirus*, *Topocuvirus*, and *Turncurtovirus*, only comprise viruses possessing monopartite genomes. Viruses in the well-studied genus, *Begomovirus*, can have either a single genome (monopartite) or two (bipartite) (Venkataravanappa *et al.*, 2019).

The bipartite begomovirus contains two encapsulated DNA molecules, designated DNA-A and DNA-B, of sizes ranging from 2.6 kb and 2.8 kb, respectively (Nawaz-ul-Rehman & Fauquet, 2009). The DNA-A of the monopartite begomovirus is structurally and genetically identical to that of the bipartite begomovirus. DNA-A and DNA-B components of bipartite begomovirus are required for successful systemic infection, where DNA-A provides transcription and replication functions and DNA-B offers movement functions (Rojas *et al.*, 2005). Monopartite is commonly associated with three satellites, namely, alpha satellite (1.3–1.4 kb), beta satellite (1.3 kb), and delta satellite or non-coding satellite (0.7 kb). Based on phylogenetic relationships, begomoviruses can be further divided into old world (OW), new world (NW), legumoviruses, and sweepoviruses (Sanchez-Chavez *et al.*, 2020). The NW originates from America, and the OW originates from Africa, Europe, Asia, and Oceania. Most NW begomoviruses are bipartite with few exceptions, whereas the OW can be either monopartite or bipartite, but predominantly monopartite (Macedo *et al.*, 2018; Torres-Herrera *et al.*, 2019). Sweepoviruses and legumoviruses are classified according to the specific host that they infect. Legumoviruses are restricted to leguminous plants in the old world. In contrast, sweepoviruses infect sweet potatoes and other members of the *Convolvulaceae* family (Briddon *et al.*, 2010; Lozano *et al.*, 2016).

The lack of begomovirus resistance in high-yielding hybrids is the main limiting factor for cucumber production in Malaysia (Goundar *et al.*, 2022). Typical symptoms of begomoviruses on cucumber are chlorosis, leaf yellowing, leaf curling, leaf crinkling, leaf distortion, leaf mottling, vein swelling, shortened internodes, and plant stunting (Sanchez-Chavez *et al.*, 2020; Chen *et al.*, 2021; Goundar *et al.*, 2022). Yield loss contributed by begomoviruses is estimated to be 18–100% depending on the environment, host, stage of infection, and virus strains (Dasgupta *et al.*, 2003). The severity of symptoms caused by begomoviruses is determined by their evolutionary fitness, which is primarily attained through recombination, mutation, and pseudo-recombination (reassortment) (Seal *et al.*, 2006). Over 15 distinct begomovirus species that infect cucurbits were found using nucleotide sequence identity thresholds of 91% (Zerbini *et al.*, 2017). In Asia, cucurbit leaf curl disease is often caused by three begomoviruses: ToLCNDV, SLCCNV, and squash leaf curl Philippines virus (SLCuPV) (Chan *et al.*, 2019). All of them possess a bipartite genome (DNA-A & DNA-B). ToLCNDV was detected in more than 15 cucurbit crops grown across nine countries, including Malaysia (Zaidi *et al.*, 2017; Chan *et al.*, 2019; Chen *et al.*, 2021).

Accurate identification of pathogens at an early stage is essential for developing an effective management strategy and designing a resistance breeding program. Observing symptoms and vectors may help diagnose, however, many viruses or nutrient deficiencies cause identical symptoms, and whiteflies carry multiple viruses, not just begomovirus. Therefore, it is important to use the most appropriate diagnostic tests to confirm the presence of begomovirus. Sequencing of PCR-amplified DNA fragments allows for comparison with other known begomovirus sequences and can aid in the accurate identification of reported as well as novel (uncharacterized) begomovirus (Inoue-Nagata *et al.*, 2004). Begomoviruses have been found in cucumbers worldwide, including Southeast Asia. Unfortunately, in Malaysia, surveillance and detection of begomovirus in cucumbers are still limited, especially in Terengganu state. Identification of begomovirus species is crucial for designing an effective resistant breeding strategy. It enables breeders to develop resistant cultivars that specifically target the identified virus species, understand the genetic variation within the virus population, tailor screening and testing protocols, and deploy resistant varieties in a targeted manner. Thus, this study aimed to confirm the presence of begomovirus using polymerase chain reaction (PCR), identify the begomovirus species, and define the evolutionary relationships among circulating begomoviruses using phylogenetic analysis.

MATERIALS AND METHODS

Plant materials

Samples of leaves were obtained from cucumber plants that were naturally infected and exhibiting begomovirus symptoms at the Green World Genetics (GWG) research station (samples C1–C6), and Lembah Bidong Farm (samples SA, SB, & SC), Rhu Tapai, Setiu, Terengganu. The leaf's midrib was

Table 1. Universal primers used in this study

Sequence Name	Sequence 5' - 3'	Annealing temperature	Source
BegA-F294	TATGBCGAAGCGWBCHRYMGA	52 °C	Charoenvilaisiri <i>et al.</i> (2020)
BegA-R1313	TTCAAYYACACCTSMGGARRG	52 °C	Charoenvilaisiri <i>et al.</i> (2020)
BegA-F1293	CYYTCCKSAGTTGTRRTTGAA	55 °C	Charoenvilaisiri <i>et al.</i> (2020)
BegA-R315	TCKRYDGVWCGCTTCGVCATA	55 °C	Charoenvilaisiri <i>et al.</i> (2020)
M9A-F2602	ATGGGTGTCTCTCCAAAACCT	52 °C	Charoenvilaisiri <i>et al.</i> (2020)
M9A-R633	TTCCCAGCACATAGATAGAT	52 °C	Charoenvilaisiri <i>et al.</i> (2020)
M9A-F1800	GCTTTAGATAGTGGGGATCAA	55 °C	Charoenvilaisiri <i>et al.</i> (2020)
M9A-R2300	CGAACATTTCAGGGAGCTAAGTC	55 °C	Charoenvilaisiri <i>et al.</i> (2020)
M9A-F973	CCTGTACTCACGCATCTAAT	55 °C	Charoenvilaisiri <i>et al.</i> (2020)
M9A-R1516	TCAGACGCAGACGAGTTGAT	55 °C	Charoenvilaisiri <i>et al.</i> (2020)
ND-DNAB-Find-F	TCCAAACAATATACGCCTAAGG	52 °C	Ito <i>et al.</i> (2008)
ND-DNAB-Find-R	TCTCAAGGATAAGAATCCTTGG	52 °C	Ito <i>et al.</i> (2008)
Beta01	ACTACGCTACGCAGCAGCC	50 °C	Kesumawati <i>et al.</i> (2020)
Beta02	TACCCTCCCAGGGGTACAC	50 °C	Kesumawati <i>et al.</i> (2020)

DNA-A sequencing, editing, and assembly

The PCR products from sample C3 were sent to Apical Scientific Sdn. Bhd. for sequencing. The DNA-A sequences were then edited manually, aligned, and assembled using Molecular Evolutionary Genetics Analysis (MEGA11) software. To search for sequence similarity and identity, the consensus sequence was blasted using the Basic Local Alignment Search Tool (BLAST) available at the National Centre for Biotechnology Information (NCBI).

Phylogenetic analysis

Maximum Parsimony (MP) and Neighbour Joining (NJ) techniques were used in phylogenetic analysis (Saitou & Nei, 1987; Hein, 1990). The MP was performed by a heuristic search of 100 replicates with the factory settings of bisection-reconnection (TBR) branch swapping, keeping only 10 trees per replication, and gaps were treated as missing values (Fatimah *et al.*, 2011). The bootstrap consensus of the tree was derived from 100 replicates. According to Kress *et al.* (2002), bootstrap percentages between 50 and 70 were considered weak, 71 to 85 as moderate, and more than 85 as strong. The analyses were performed using MEGA11 software (Tamura *et al.*, 2021).

RESULTS AND DISCUSSION

Begomovirus detection

The primers M9A-F1800/M9A-R2300 successfully amplified a single band at approximately 500 bp in all samples (Figure 2), confirming the presence of the begomovirus DNA-A genome. This primer set was designed to target only the partial Rep protein. The Rep ORF is vital in viral transcription stimulation and is required for the initiation, elongation, and termination of the viral replication process (Fondong, 2013; Snehi *et al.*, 2017). It also exhibits sequence-specific DNA binding ability, endonuclease (site- & strand-specific), and ligase activity in almost every geminivirus examined thus far (Ruhel & Chakraborty, 2019). Therefore, this primer set can be considered the best option to amplify the begomovirus-infected samples in future work.

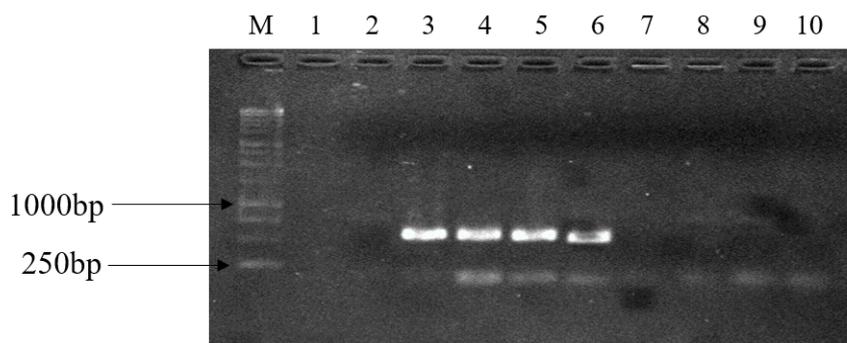


Fig. 2. A single banding (around 500 bp) pattern produced by primers M9A-F1800/M9A-R2300. (M) = 1kb marker, (2) = C1, (3) = C2, (4) = C3, (5) = C4, (6) = C5, (7) = C6, (8) = SA, (9) = SB, (10) = SC.

In addition, a single band was amplified by primers M9A-F973/M9A-R1516 at an estimated size of 540 bp (Figure 3), targeting the partial TrAP, entire REn, and partial CP regions. Out of nine samples, a clear single band was amplified only from samples C2-C5. REn is not necessary for virus replication but increases viral DNA accumulation and symptom development in begomovirus-infected plants (Sunter *et al.*, 1990; Sung & Coutts, 1995). Therefore, the Rep gene can replicate and transcribe viral DNA with or without REn (Snehi *et al.*, 2017). Moreover, TrAP, Ren, and movement proteins are not virus-specific and can trans-complement defects in other begomoviruses (Frischmuth *et al.*, 1993; Saunders & Stanley, 1995). A 15-kDa protein encoded by the AC2/TrAP gene is necessary for viral replication, transcriptional control of late viral genes, and host defense suppression, including gene silencing (Guerrero *et al.*, 2020).

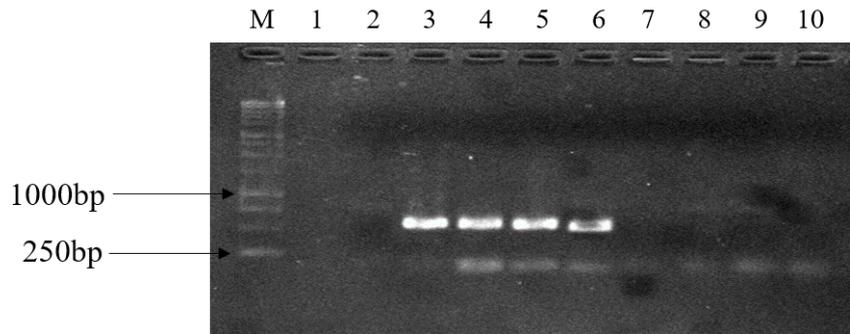


Fig. 3. A single banding (540 bp) pattern produced by primers M9A-F973/M9A-R1516. (M) = 1kb marker, (2) = C1, (3) = C2, (4) = C3, (5) = C4, (6) = C5, (7) = C6, (8) = SA, (9) = SB, (10) = SC.

The primers BegA-F294/BegA-R1313 produced a single band at an estimated size of 1000 bp in samples C1 and C2 (Figure 4). This primer targets the entire CP region, partial REn, and partial pre-coat protein (PCP) regions. The coat/capsid protein (CP) is from the late gene V1/AV1, and the sole structural protein found in geminivirus particles is CP, which is capable of encapsulating the genome. The CPs of monopartite begomoviruses are necessary for systemic movement within the plant and can bind both ss and dsDNA (Bridson *et al.*, 1989; Lazarowitz *et al.*, 1989; Liu *et al.*, 1997). The only virus protein to which whitefly tissues have been exposed and are available to interact with insect receptors or other proteins that facilitate or assist in virus translocation in insect tissues is CP. Although all begomovirus CPs contain highly conserved sequences, they also contain variable regions (Fondong, 2013). Only old-world viruses possess the PCP gene (V2/AV2). It is part of the virion strand and shares 190–200 bases of DNA with the CP gene. PCP is necessary for pathogenicity, systemic spread, and suppression of gene silencing in the host (Roshan *et al.*, 2018). As the PCP sequence is recombination-prone, it has high sequence variability. As PCP and CP overlap, the overall sequence variability of the CP gene will be significantly influenced by the presence of a diverse PCP gene (Mondal *et al.*, 2019).

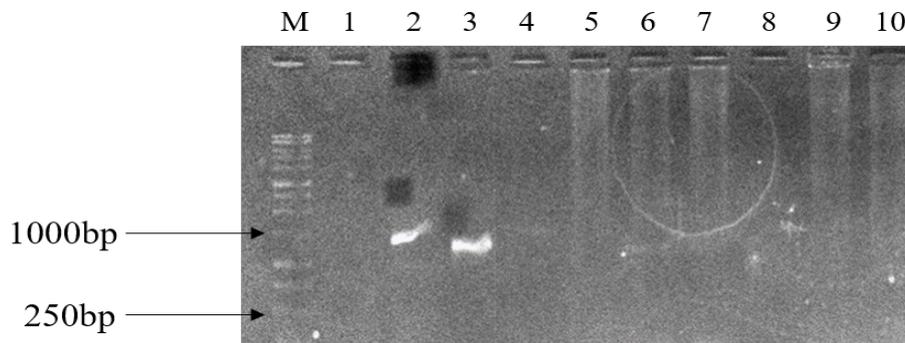


Fig. 4. A single banding (around 1000 bp) pattern produced by primers BegA-F294/BegA-R1313. (M) = 1kb marker, (2) = C1, (3) = C2, (4) = C3, (5) = C4, (6) = C5, (7) = C6, (8) = SA, (9) = SB, (10) = SC.

Nonetheless, the primers BegA-F1293/BegA-R315 and M9A-F2602/M9A-R633 failed to produce any amplified band for the DNA-A genome, while the primers ND-DNAB-Find-F/ND-DNAB-Find-R do not amplify any band for the DNA-B genome (data not shown). For the beta satellite, the primers Beta01 and Beta02 were used to amplify PCR products with an estimated size of 1345 bp. Out of nine samples,

only one (SB) produced a clear single band (Figure 5). In general, beta satellites (& also alpha satellites) are found associated with the old-world (OW) monopartite begomoviruses (Briddon & Stanley, 2006; Zhou, 2013; Sohrab *et al.*, 2017) but not in bipartite begomoviruses with few exceptions reported so far, Mungbean yellow mosaic India virus (MYMIV) in mungbean (Rouhibakhsh *et al.*, 2005; Qazi *et al.*, 2007), Tomato leaf curl New Delhi Virus (ToLCNDV) in tomato (Sivalingam *et al.*, 2010) and spine gourd (Venkataravanappa *et al.*, 2019). In this study, for the first time, we have identified ToLCNDV (a bipartite begomovirus) associated with a beta satellite in cucumber. Betasatellites are trans-replicated by geographically divergent and biologically diverse other helper begomoviruses and mastreviruses (Saunders *et al.*, 2008). For trans-replication, betasatellites can form promiscuous connections with various begomoviruses, unlike the replication of DNA A and DNA B specifically by cognate Rep. Betasatellite genome has a unique "Rep binding motif" (RBM) that exhibits distinct binding affinities with cognate and non-cognate Rep proteins. This is attributed to the trans-replication efficiency and promiscuous selection of several betasatellites (Briddon *et al.*, 2003; Tao *et al.*, 2004). Hence, if beta satellite molecules come across new begomoviruses from diverse geographical origins, they might give rise to new begomovirus-beta satellite complexes (Sattar *et al.*, 2019). The growing incidence of ToLCNDV and other bipartite begomoviruses in connection with betasatellites is likely due to co-infections between bipartite and monopartite begomoviruses that require betasatellites (Iqbal *et al.*, 2017). The relationship between bipartite and beta satellites has not been fully studied, which confirms that DNA-B prevents or interferes with the interaction between DNA-A and a satellite (Briddon *et al.*, 2010).

DNA-A sequence analysis

A partial DNA-A sequence was isolated from cucumber leaf sample C3 and amplified by the primers M9A-F973/M9A-R1516. Table 2 shows that the query coverage of this partial DNA-A sequence was 100% overlapped with the ToLCNDV reference sequence, which is consistent with the report of Kesumawati *et al.* (2020). The partial sequence had the highest similarity (97.67%) with the Tomato leaf curl New Delhi virus (ToLCNDV) isolated from cucumber in Thailand (AB330079), followed by 97.59% similarity with muskmelon in Thailand (AB368448), 97.42% with cucumber in Thailand (MK883715), 97.16% with tomato in Bangladesh (KM383744), and 96.90% with tomato in India (MT316390).

Phylogenetic analysis

DNA-A is more conserved than DNA-B, so it is often used for phylogenetic analysis in begomoviruses. In general, DNA-B has a greater diversity than DNA-A, with fewer conserved elements. The reason for more diversity in DNA-B might be that it is less congested (coding for fewer proteins without overlapping genes), and has exclusive evolution in response to the host (in contrast, DNA-A needs to interact with both vector & host), or has a completely distinct evolutionary path. The contribution of DNA-B to the species level is little, but it can play a major role at the strain level. Hence, if one species has two isolates, each with unique DNA-B, they might be referred to as different strains (Harrison & Robinson, 1999; Briddon *et al.*, 2010). During pseudo-recombination, the process by which DNA-A catches DNA-B is referred to as "regulon grafting". DNA-A contributes its common region (CR) to DNA-B, which is captured through recombination and establishes a new dependency between the two components (Saunders *et al.*, 2002). Also, a similar donation of replication origin has been discovered for the beta satellites associated with begomoviruses (Saunders *et al.*, 2001).

For the phylogenetic analysis of begomoviruses, the outgroup Pepper huasteco yellow vein virus (PHYVV) was selected because this virus is closely related to the group being studied (Retes-Manjarrez *et al.*, 2019). Both NJ and MP phylogenetic trees showed a similar distribution of samples (Figures 6 & 7). The trees were grouped into two (group A & group B). In group A, the partial DNA-A sequence from sample C3 showed the closest relationship with the Tomato leaf curl New Delhi virus (ToLCNDV), and their relationship was considered sister because they split from the same node and were strongly supported with 100% bootstrap. Both C3 and ToLCNDV were closely related to Squash leaf curl China virus (SLCCNV) with 100% bootstrap support, followed by Ageratum yellow vein virus (AYVV) with 99% bootstrap support in the NJ tree and 68% bootstrap support in the MP tree. In group B, the Tomato yellow leaf curl Kanchanaburi virus (TYLCKaV) showed strong support with 100% bootstrap to the Pepper yellow leaf curl Indonesia virus (PepYLCIV), followed by the Pepper yellow leaf curl Aceh virus (PepYLCAV). TYLCKaV, PepYLCIV, and PepYLCAV are bipartite begomoviruses (Kesumawati *et al.*, 2019).

Though ToLCNDV, SLCCNV, and AYVV are closely related, ToLCNDV and SLCCNV are bipartite begomoviruses, whereas AYVV is a monopartite begomovirus (Zaidi *et al.*, 2017; Wu *et al.*, 2022).

This is because begomoviruses can swiftly evolve through recombination, pseudo-recombination, mutation, and the acquiring of new DNA components and satellites. Bipartite begomoviruses arose from monopartite that gained a DNA-B component, which was most likely derived from the initial DNA-A (Materatski *et al.*, 2021). In natural conditions, monopartite begomoviruses have been observed to acquire DNA-B during mixed infections. This acquisition leads to the formation of bipartite begomoviruses, which are specifically differentiated as mono-bipartites. The transition from monopartite to bipartite begomoviruses is considered an evolutionary adaptation that allows the virus to overcome certain constraints and enhance its ability to infect and spread in new hosts or environments (Saunders *et al.*, 2002; Chakraborty *et al.*, 2003).

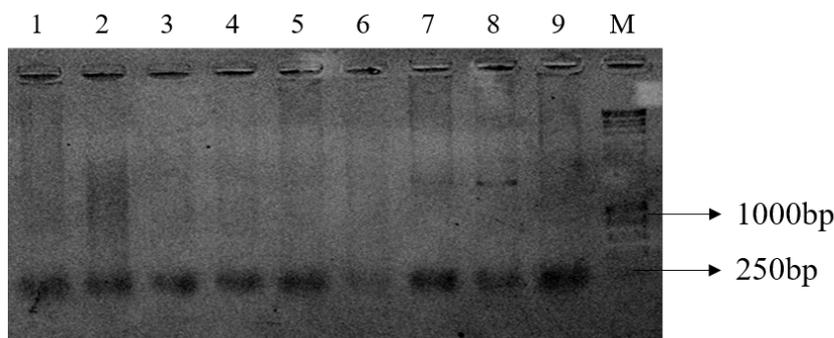


Fig. 5. A single banding (1345 bp) pattern produced by primers Beta01/Beta02. (1) = C1, (2) = C2, (3) = C3, (4) = C4, (5) = C5, (6) = C6, (7) = SA, (8) = SB, (9) = SC, (M) = 1kb marker.

Table 2. Similarity and identity of partial DNA-A sequence of begomovirus obtained from BLAST

DNA molecule and size (bp)	Host	Query coverage %	Identity %	Virus	Accession number	Reference
DNA-A sample-C3 (1169)	<i>Cucumis sativus</i> (Cucumber) from Malaysia	100	100	ToLCNDV	-	This study
DNA-A (2739)	<i>Cucumis sativus</i> (Cucumber) from Thailand	100	97.67	ToLCNDV	AB330079	Ito <i>et al.</i> (2008)
DNA-A (2739)	<i>Cucumis melo</i> (Muskmelon) from Thailand	100	97.59	ToLCNDV	AB368448	Ito <i>et al.</i> (2008)
DNA-A (2739)	<i>Cucumis sativus</i> (Cucumber) from Thailand	100	97.42	ToLCNDV	MK883715	Lee <i>et al.</i> (2020)
DNA-A (2739)	<i>Solanum lycopersicum</i> (Tomato) from Bangladesh	100	97.16	ToLCNDV	KM383744	Akhond <i>et al.</i> (2014) Unpublished*
DNA-A (2739)	<i>Solanum lycopersicum</i> (Tomato) from India	100	96.90	ToLCNDV	MT316390	Patel <i>et al.</i> (2020) Unpublished*

* Source: National Centre for Biotechnology Information (NCBI)

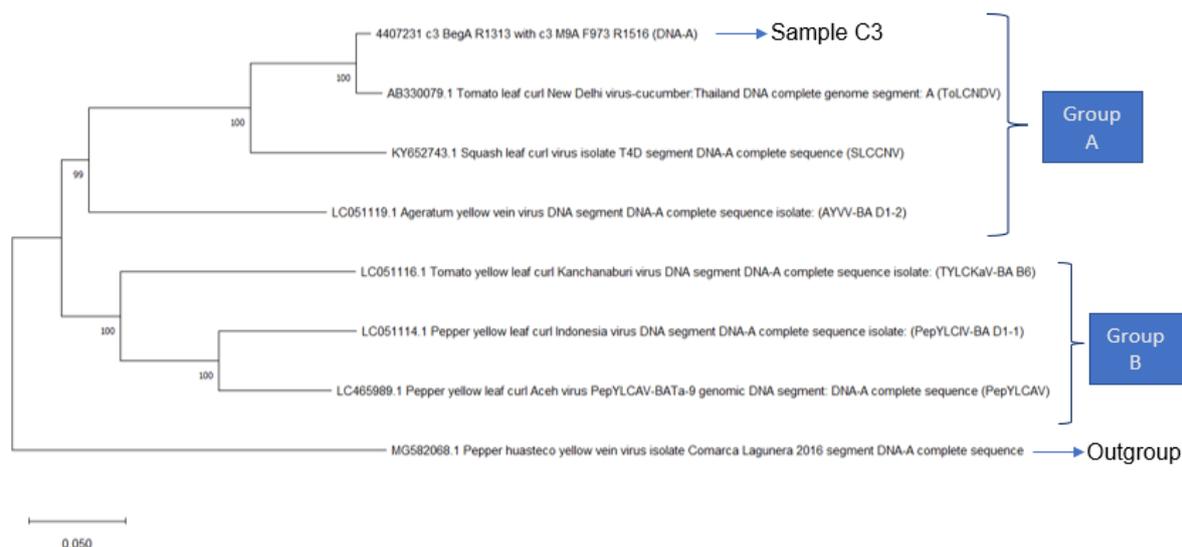


Fig. 6. Phylogenetic tree constructed based on the neighbor joining method (Saitou & Nei, 1987).

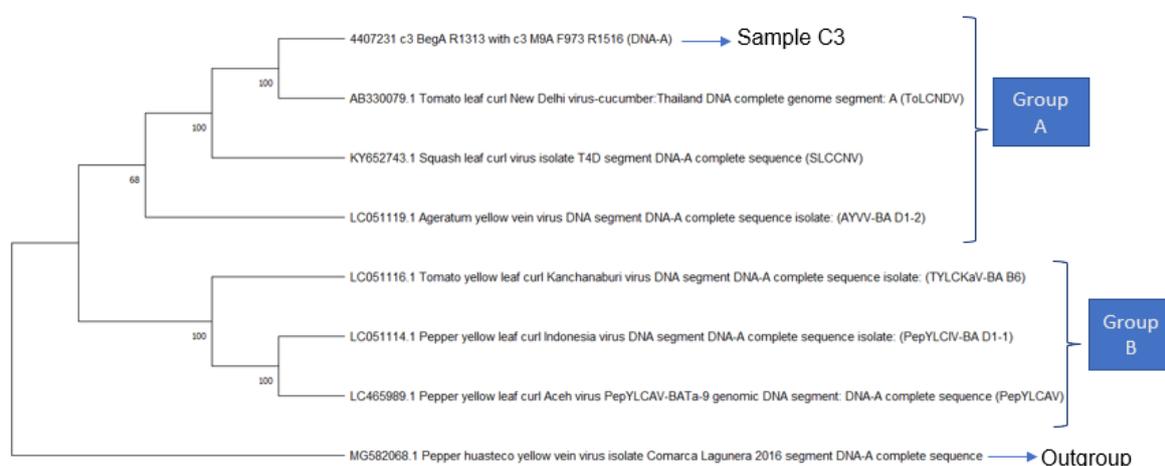


Fig. 7. Phylogenetic tree constructed based on the maximum parsimony method (Hein, 1990).

CONCLUSION

The presence of begomovirus on symptomatic cucumber crops collected from the Green World Genetics (GWG) research station and a cucumber farm in Lembah Bidong, Rhu Tapai, Setiu district, Terengganu, was confirmed by PCR. The partial DNA-A sequence of begomovirus isolated in this study (C3) was 100% identical to ToLCNDV, and their relationship was also strongly supported as a sister by 100% bootstrap. However, ToLCNDV is a bipartite virus; therefore, further investigation of the DNA-B sequence is needed. To the best of our knowledge, this is the first report of a beta satellite associated with ToLCNDV, a bipartite OW begomovirus in cucumber. The presence of the DNA-B component hinders or disrupts the interaction between the DNA-A component and a satellite; hence, studying a beta satellite associated with a bipartite begomovirus will be even more challenging than studying a monopartite associated with a beta satellite. In the future, a complete genome sequence of the begomovirus infecting cucumber in Malaysia is required. The findings of this study contribute significantly to our knowledge of the begomovirus disease complex and the development of begomovirus-resistant cucumber varieties in the future.

ACKNOWLEDGEMENTS

The authors are thankful to Universiti Sultan Zainal Abidin (UniSZA) for granting this study through Dana Penyelidikan Universiti 2.0 (UniSZA/2023/DPU 2.0/24). We also thank Dr. Chua Kim Aik, the CEO of Green World Genetics, for funding the Ph.D project of Sakthivel Poraya Goundar.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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