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Identification of Garlic Viruses Associated with Seed Bulbs and Consumption Bulbs from Several Locations in Indonesia

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ABSTRACT

Virus infection is one of the major constraints in garlic production since the viruses are readily accumulated on vegetative propagation material (bulbs). This research aimed to detect garlic common latent virus (GCLV), shallot latent virus (SLV), onion yellow dwarf virus (OYDV), and leek yellow stripe virus (LYSV) infecting local garlic as seed bulb and imported garlic as consumption bulb. Seed bulb samples were obtained from seed breeders in several garlic growing centers in Indonesia. In contrast, consumption bulb samples were obtained from plant quarantine warehouses and three local markets in Bogor. Some bulb samples were used for morphological observations, and some were germinated in the laboratory until the leaves emerged. Leaves were collected for virus detection by RT-PCR using specific primers for GCLV, SLV, OYDV, and LYSV. Seed and consumption bulbs have differences in their morphological characteristics, especially in the type of neck hardness and the size of the bulb diameter. OYDV and LYSV infections were successfully detected in seed and consumption bulbs, while SLV was only found in consumption bulbs. Nucleotide sequence analysis showed that SLV from consumption bulbs formed one group, GCLV from seed bulbs formed one group, while OYDV and LYSV from seed and consumption bulbs were in different groups, indicating that the viruses came from different strains. Further research through high-throughput detection methods and providing virus-free planting material are needed to anticipate the spread of new strains of garlic viruses in Indonesia.

1. Introduction

Garlic is one of the economically important commodities globally, including for Indonesians, due to its importance in both culinary and human health. According to the Ministry of Agriculture (2020), domestic garlic consumption for the period 2020-2024 is estimated to increase by 1.38% per year. The increase in garlic consumption in Indonesia is different from its production. The largest garlic-producing provinces in Indonesia are Central Java, West Nusa Tenggara, and East Java. Based on BPS (2023), garlic production in Indonesia in 2021 reached 45,092 tons, a decrease of 44.88%. In order to meet domestic consumption needs, Indonesia imports garlic from other countries, and this contributes to almost 95% of garlic on the market (Feryanto *et al.* 2022).

One of the constraints on the low production of garlic in Indonesia is the infection caused by viruses. The main viruses infecting garlic are Garlic common latent virus (GCLV) and Shallot latent virus (SLV), members of the genus Carlavirus and Onion yellow dwarf virus (OYDV) and Leek yellow stripe virus (LYSV), members of the genus Potyvirus (Katis *et al.* 2012). GCLV, SLV, OYDV, and LYSV are single-stranded RNA viruses and are commonly found infecting garlic together as garlic virus complexes (Cremer *et al.* 2021). Its infection causes yellow mosaic stripes, streaking, and curling on the leaf (Parrano *et al.* 2012; Cremer *et al.* 2021). GCLV, SLV, OYDV, and LYSV are seed (bulbs)-transmitted viruses (Katis *et al.* 2012). Thus, the use of pathogen-free seed bulbs, especially viruses, is highly recommended to maintain crop productivity. Field monitoring and selection of virus-free seeds require an accurate virus detection method. To date, the use of serological detection method has been used as one of the common methods for *Allium* virus indexing in Indonesia due to its efficiency, even though

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this technique has limitations on the availability of commercial antiserum and sensitivity (Harti *et al.* 2020; Hidayat *et al.* 2023). The RT-PCR (reverse transcription polymerase chain reaction) is one of the virus detection methods that has high sensitivity because this assay can detect viruses in low concentrations (Lunello *et al.* 2005).

Previous studies on viruses infecting garlic plants in Indonesia still need to cover a lot of information about virus infection in seed and consumption bulbs. The use of RT-PCR has been conducted to detect OYDV, LYSV, and GCLV from commercial and non-commercial garlic cultivars (seed bulbs) in Indonesia (Hidayat *et al.* 2023). In fact, the introduction of imported garlic to Indonesia has a big potential as the entrance for new emerging viruses. Therefore, this study was conducted to detect the main viruses infecting local garlic bulbs (as seed bulbs) and imported garlic bulbs (as consumption bulbs) using the RT-PCR method. Data and information from the detection results can be used as a basis to develop a prevention and disease control strategy for viruses infecting garlic in Indonesia.

2. Materials and Methods

2.1. Local Garlic Germplasm Collection

Garlic samples consisted of two types, i.e., seed and consumption bulbs. Local seed bulbs were obtained from (i) bulb breeders in Central Java Province (Tawangmangu District, Karanganyar Regency) and West Nusa Tenggara Province (Sembalun District, East Lombok Regency) and (ii) the germplasms collection of the Tissue Culture Laboratory, Department of Agronomy and Horticulture, IPB University. Consumption bulbs (imported bulbs) were purchased from three traditional markets in Bogor, namely 'The Central Kemang market, The Anyar market, and 'The Sukasari market, and also provided by Indonesian Plant Quarantine from a quarantine warehouse in Jakarta.

2.2. Observation of Bulb Morphology and Symptoms of Virus Infection

Bulb samples from each location were selected randomly. The morphological character of each bulb was observed, including skin color, neck hardness, and diameter size. Each selected bulb was broken, and then each clove was planted on a plastic tray filled with styrofoam and water. Symptoms were observed two weeks after planting (WAP) and documented using a digital camera. The garlic leaves were harvested at 4 WAP and used as material for virus detection using the RT-PCR method.

2.3. Virus Detection by RT-PCR Method

Total RNA extraction was performed using a GeneJET RNA Purification kit (Thermo-Fisher Scientific, Waltham, US). Amplification was done using a one-step RT-PCR method. Each 25 µl of RT-PCR mixture consisted of 12.5 µl DreamTaq green master mix (2×) (Thermo-Fisher Scientific, Waltham, US); 1 µl 0.1 M DDT; 0.25 µl RT enzyme (Smobio, Taiwan); 0.5 µl Ribolock/RNase inhibitor (Bioline, London, UK); 2 µl total RNA; 2 µl each of 10 µM forward and reverse primers of OYDV, LYSV, SLV, and GCLV (Table 1) (IDT, Singapore); and added water up to get a final volume. The initial stage of cDNA synthesis was carried out at 45°C for 60 min. The next step was cDNA amplification, consisting of pre-denaturation at 94°C for 1 min, followed by 35 cycles consisting of denaturation at 94°C for 30 sec, annealing at 50°C for 30 sec, and elongation at 72°C for 1 min. The last step is the final elongation cycle at 72°C for 10 min. Representative positive samples were then sent to First Base Malaysia for nucleotide sequencing.

2.4. Nucleotide Sequence Analysis

The nucleotide sequences were trimmed and assembled using Geneious Prime software (Biomatters, Ltd., Auckland) to get a contig sequence. The BLAST

Table 1. Primers used for amplification of onion yellow dwarf virus (OYDV), leek yellow stripe virus (LYSV), shallot latent virus (SLV), and garlic common latent virus (GCLV)

Virus target	Primer codes	Primer sequences (5′–3′)	Target size (bp)	Target region*	References
OYDV	OG-RT1	GAAGCGCACATGCAAATGAAG	290	Partial CP	Sumi et al. (2001)
	OG-RT2	CGCCACAACTAGTGGTACAC			
LYSV	P-RT3	AAGAGTCAACACTTGGTTTG	191	3′-UTR	Haque and Hattori (2017)
	P-RT4	GGTCTCAATCCTAGCTAGTC			
SLV	GS-RT1	TATGCTCGAGCTCGTAGAGC	170	Partial NABP	Haque and Hattori (2017)
	GS-RT2	GGGTTTCACATTGTTACACC			
GCLV	GCLV-F	ATGTCAGTGAGTGAAACAGAGG	960	CP	Parrano *et al.* (2012)
	GCLV-R	CTAGTCTGCATTGTTGGATCC			

*CP, coat protein; UTR, untranslated region; NABP, nucleic acid binding protein

program (https://blast.ncbi.nlm.nih.gov/Blast.cgi) was used to search for matches to related virus sequences from each contig sample. Selected sequences with high similarity were then analyzed using ClustalW multiple alignments in MEGA X software (Kumar *et al.* 2018). The alignment results were then analyzed using the sequence demarcation tools (SDT) Matrix (Muhire *et al.* 2014) to visualize the homology level of each virus target (OYDV, LYSV, SLV, and GCLV).

3. Results

3.1. Morphology of Seed and Consumption Bulbs

The samples of seed bulbs composed of different local garlic varieties, i.e., 'Lumbu Hijau,' 'Sangga Sembalun,' 'Siam,' 'Lumbu Kuning,' and 'Ciwidey,' while the variety and origin of imported bulbs from the market were unknown. Seed and consumption bulbs have differences in morphological characteristics, including skin color, neck hardness, and diameter size. All consumption bulbs have white bulb skin color, while for seed bulbs, there are varieties that have purple bulb skin color, such as 'Lumbu Hijau' and 'Sangga Sembalun.' In general, consumption bulbs have larger diameter than seed bulbs, with an average diameter of 60.3 mm to 63.5 mm. The average diameter of seed bulbs is 38.4 mm for 'Sangga Sembalun,' and 42 mm for 'Lumbu Hijau .'Consumption bulbs belong to the soft-neck type, while seed bulbs belong to the hard-neck type characterized by the presence of flower stalks inside the pseudo-stem when cut transversely (Figure 1).

3.2. Symptom of Virus Infection

Symptoms of virus infection were found in bulb samples grown in the laboratory. Five common symptoms were found, including abnormal leaf shape (leaf malformation), stunting, leaf curling, yellow mosaic, green mosaic, and leaf curling at the tip (Figure 2).

3.3. Types and Distribution of Viruses Detected from Germinated Bulbs

The presence of OYDV, LYSV, SLV, and GCLV infections in garlic bulb samples was successfully detected through RT-PCR (Figure 3). Detection results using specific primers for the various target viruses showed that there was mixed infection in all tested samples from both seed and consumption bulbs (Table 2). All tested samples gave positive reactions for OYDV and LYSV. SLV-infected samples were only found in the consumption bulbs. In contrast, GCLV infection was only found in seed bulbs, i.e., 'Ciwidey,' 'Siam,' and 'Sangga Sembalun,' even though in further detection, GCLV was also detected on two samples of consumption bulbs (data was not shown).

3.4. Sequence Identity and Phylogenetic Tree Analysis

Nucleotide sequence analysis was used to confirm further the identity of each virus target from positive amplicons. Nucleotide sequence analysis using the BLAST program showed that the viruses detected in local and consumed seed bulbs were confirmed as OYDV, LYSV, SLV, and GCLV. The percentage similarity of nucleotide varied between 86.48% and 100%.

Multiple sequence alignment with closely related OYDV sequences from GenBank (27 accessions) showed nucleotide identity varying from 72 to 100% (Figure 4A) and were 100% nucleotide identical with Chinese isolates (GenBank MN059602.1, AJ409311.1, AJ307033.1). Comparison of 31 isolates with closely related LYSV sequence shared nucleotide similarity ranging from 84% to 100% (Figure 4B) and had the highest level of

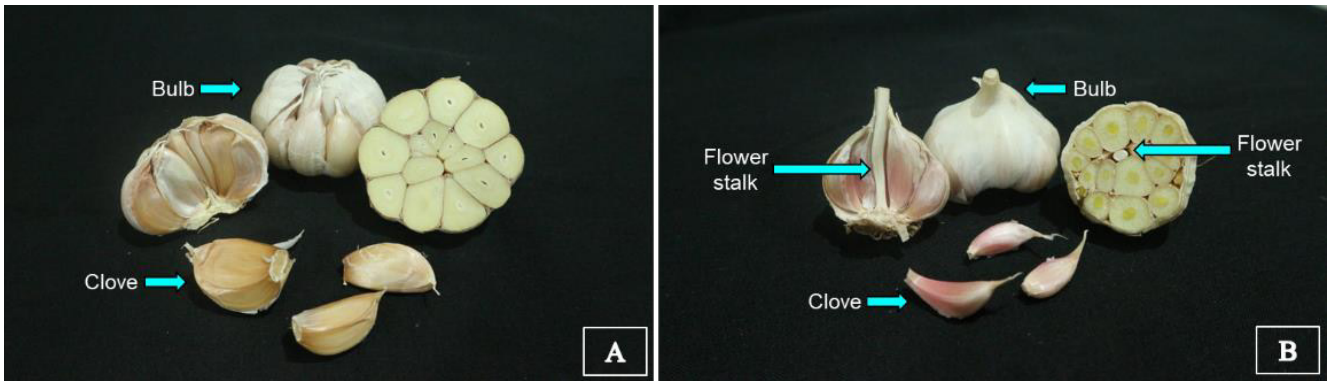

Figure 1. Garlic bulb types based on neck hardness. (A) soft-neck type of consumption bulbs; (B) hard-neck type of seed bulbs

Figure 2. Symptoms of virus infection from germinated garlic bulbs. (A) leaf malformation, (B) stunting, (C) leaf curling, (D) yellow mosaic, (E) green mosaic, and (F) leaf curling at the tip. Each symptom is indicated with a red arrow

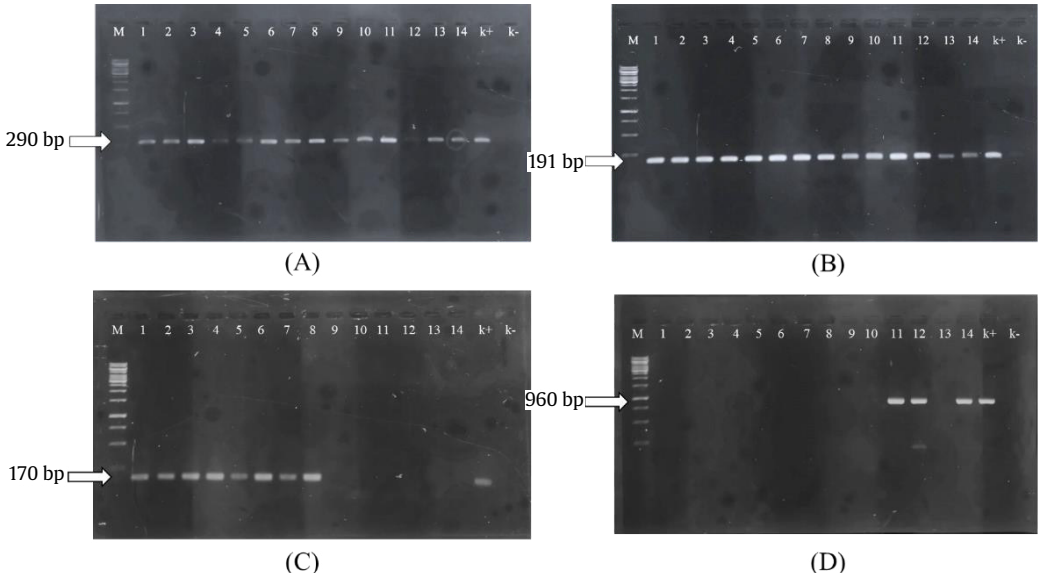

Figure 3. Visualization of DNA fragments of onion yellow dwarf virus (OYDV) (A), leek yellow stripe virus (LYSV) (B), shallot latent virus (SLV) (C), and garlic common latent virus (GCLV) (D) amplified by RT-PCR using specific primers. M, 1 kb DNA ladder (ThermoScientific, Waltham, US); 1-8, consumption bulbs; 9-14, seed bulbs; k_+, positive control; k-, negative control

Table 2. Summary of virus detection from seed and consumption bulb samples using RT-PCR method*

Sample's origin	Sample's code	Virus target**			
		OYDV	LYSV	SLV	GCLV
Seed bulbs					
Tawangmangu District, Karanganyar Regency, Central Java Province	LH1	+[a]	+	-[b]	-
	LH2	+	+	-	-
Tissue Culture Laboratory, Department of Agronomy and Horticulture, IPB University (Cianjur Regency, West Java Province)	CW1	+	+	-	-
	CW2	+	+	-	+
	SM	+	+	-	+
	LK	+	+	-	-
Sembalun District, East Lombok Regency, West Nusa Tenggara Province	SS1	+	+	-	-
	SS2	+	+	-	+
	SS3	+	+	-	+
	SS4	+	+	-	+
	LK	+	+	-	+
	LH	+	+	-	+
	LP	-	+	-	-
Consumption bulbs					
Sukasari market, Bogor Regency, West Java Province	SB5C4	+	+	+	-
	SB3C2	+	+	+	-
Anyar market, Bogor Regency, West Java Province	AB4C1	+	+	+	-
	AB6C3	+	+	+	-
	AB7C4	+	+	+	-
Central Kemang market, Bogor Regency, West Java Province	IB1C4	+	+	+	-
	IB3C5	+	+	+	-
	IB5C1	+	+	+	-
Plant Quarantine Warehouse	2964/B1/C1	+	+	+	+
	2964/B1/C5	+	+	+	+
	2964/B3/C4	+	+	-	+
	2999/B1/C1	+	+	-	-
	2999/B1/C5	+	+	-	+
	2999/B2/C1	+	+	-	+
	2999/B7/C5	+	+	-	+
	3000/B1/C1	+	+	+	+
	3000/B3/C4	+	+	-	+
	3000/B7/C5	+	+	-	+
	3003/B1/C2	+	+	-	+
	3003/B3/C2	+	+	-	+
	3003/B6/C5	+	+	-	+
	3025/B1/C2	+	+	-	+
	3025/B2/C3	+	+	-	+
	3025/B7/C4	+	+	-	+

*Partial of this data was obtained from Mawarni (2023), **OYDV (onion yellow dwarf virus), LYSV (leek yellow stripe virus), SLV (shallot latent virus), GCLV (garlic common latent virus), [a]positive reaction; [b]negative reaction

homology with LYSV isolates from Australia (GenBank JN127340.1), China (GenBank MN059525.1), and Iran (GenBank MT232838.1). For the carlavirus groups, SLV sequence analysis from 18 isolates were 77% and 100% identical (Figure 5A) and shared the highest similarity (100%) with the Chinese garlic isolate (GenBank MN059229.1). Meanwhile, 23 isolates of GCLV shared 88% to 100% nucleotide similarity (Figure 5B), and the matrix and tested samples were most closely related to the Argentinian isolate (97% nucleotide identity; GenBank KJ124848.1).

Further phylogenetic analysis of potyvirus members, OYDV, and LYSV showed the distinction between import and local garlic samples (Figures 6A and B). All imported samples were grouped in the OYDV-Garlic (OYDV-G) group, while OYDV infecting local garlic was on the OYDV-Onion (OYDV-O). For LYSV, the phylogenetic tree also fell into two major groups, and all imported garlic was in S-type (Figure 6B). Interestingly, there were variations within LYSV infecting local garlic, i.e., S-type and N-type. For carlavirus members, SLV-infecting imported garlic was in the group I (Figure 7A), while GCLV-infected seed and consumption bulbs were separated into different clades (Figure 7B).

4. Discussion

Viruses in garlic plants are generally transmitted through bulbs used for vegetative propagation. Garlic farmers in Indonesia commonly produce their seed

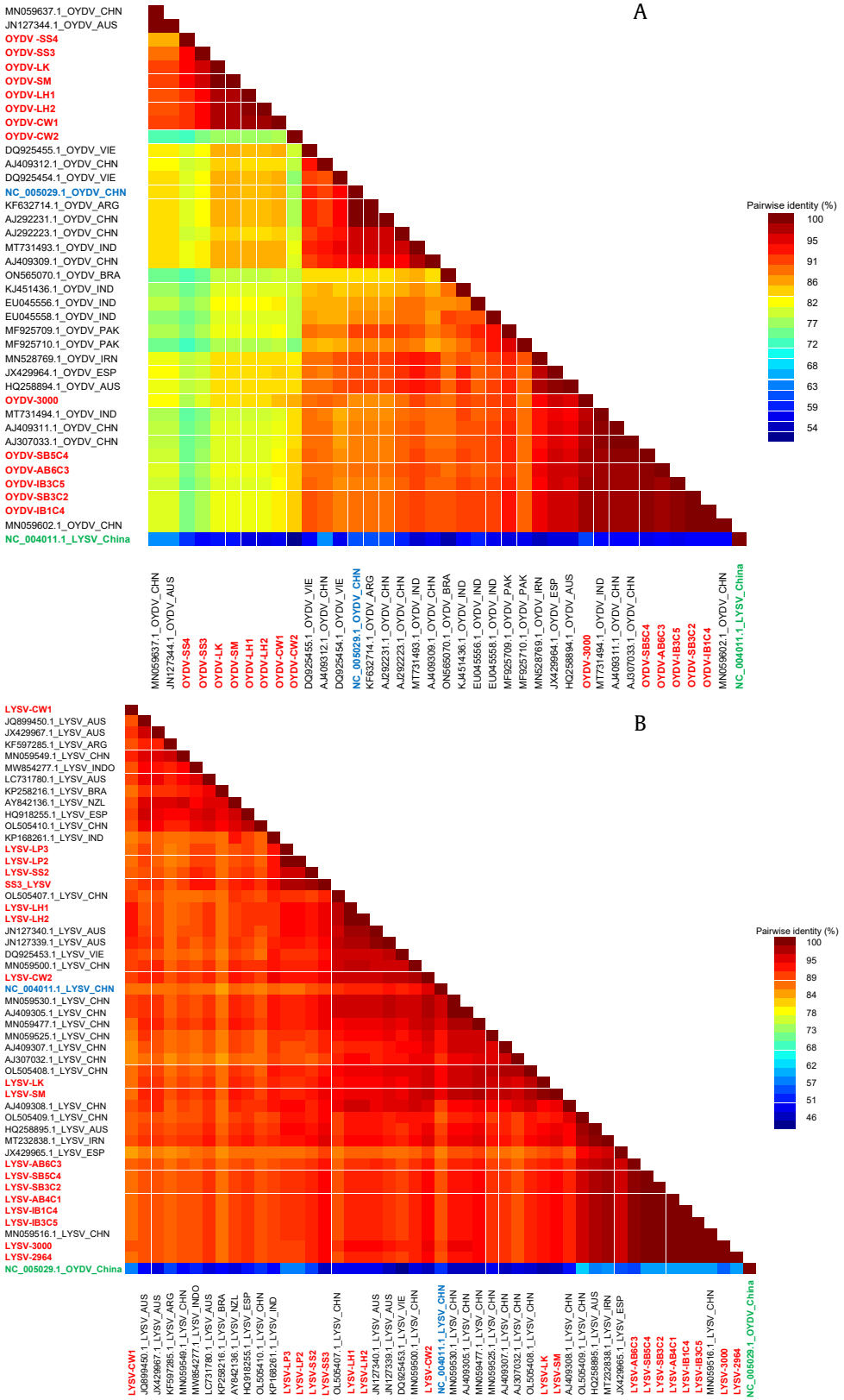

Figure 4. Sequence identity matrix of potyviruses infecting garlic, onion yellow dwarf virus (OYDV) (A) and leek yellow stripe virus (LYSV) (B). The percentage of identity was presented in color code. The tested samples, the reference sequence, and the outgroup are written in red, blue, and green letter font, respectively

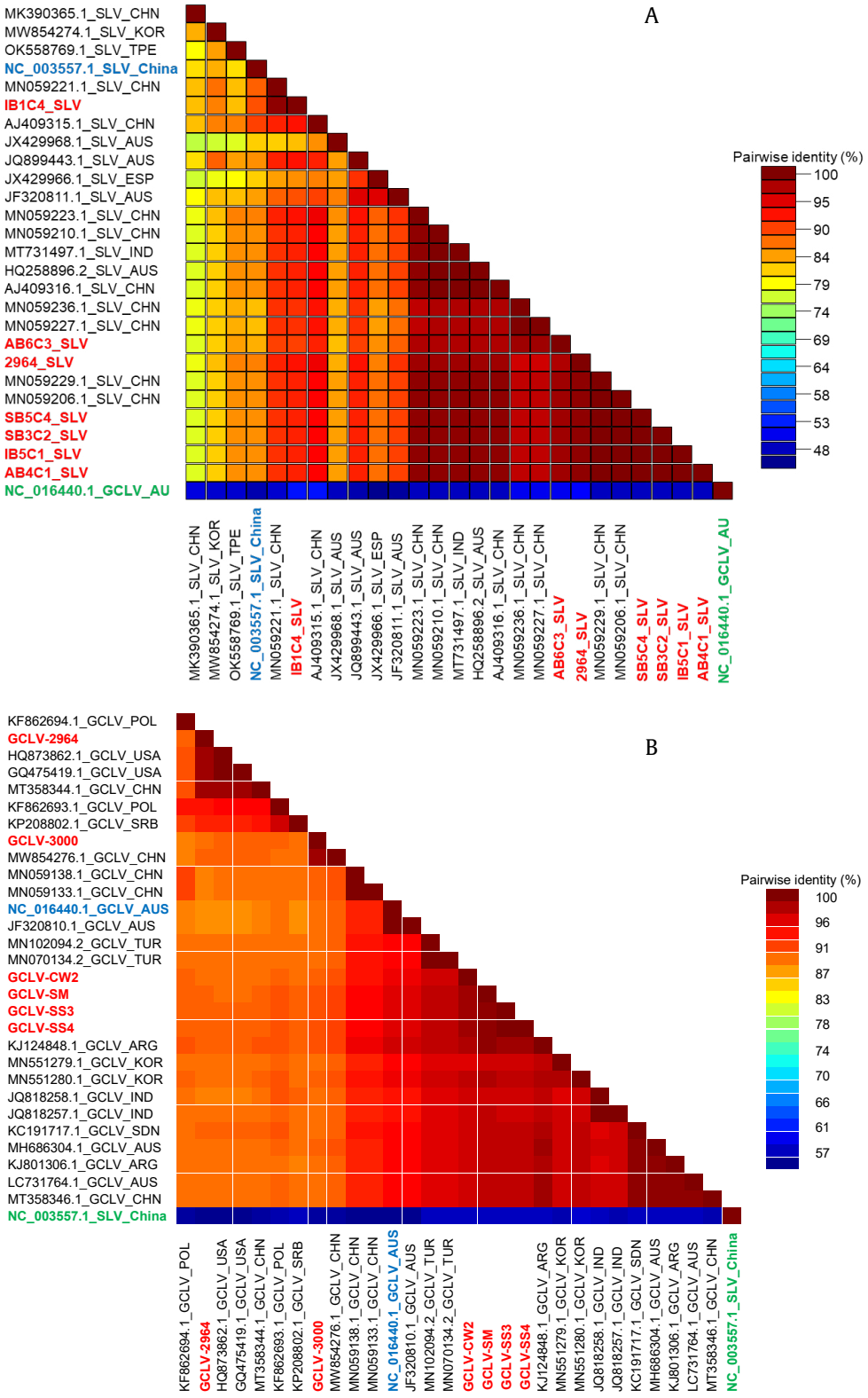

Figure 5. Sequence identity matrix of carlaviruses infecting garlic, shallot latent virus (SLV) (A) and garlic common latent virus (GCLV) (B). The percentage of identity was presented in color code. The tested sample, the reference sequence, and the outgroup are written in red, blue, and green letter font, respectively

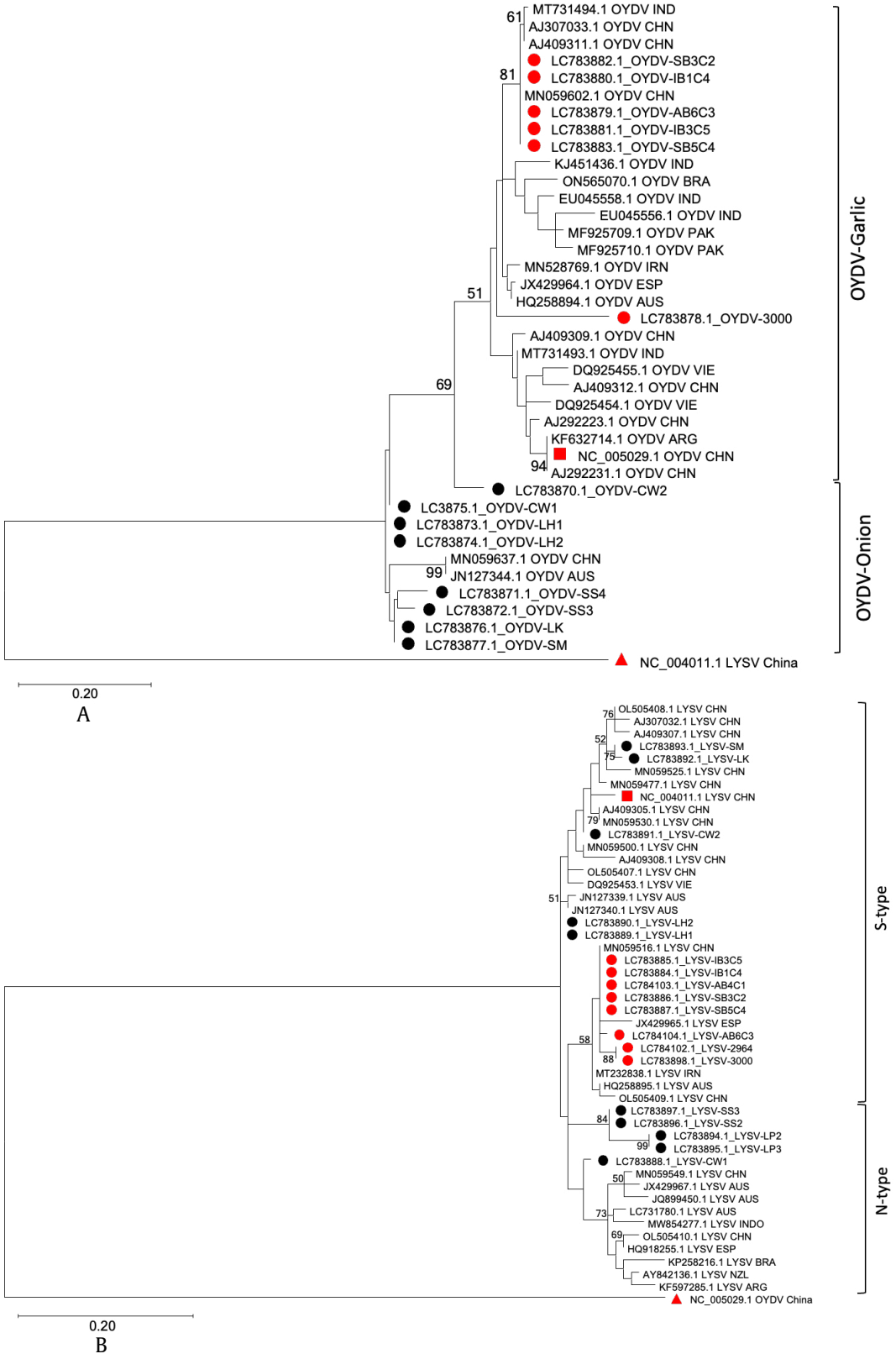

Figure 6. Maximum-likelihood phylogenetic trees of potyviruses group infecting imported and Indonesian local ga0rlic based on onion yellow dwarf virus (OYDV) (A), leek yellow stripe virus (LYSV) (B). The phylogeny was implemented with MEGA X and bootstrap with 1000 replicates. The symbol ● designates the local garlic virus isolates (from seed bulbs), ● imported garlic virus isolates (from consumption bulbs), ■ the reference sequence, and ▲ the outgroup

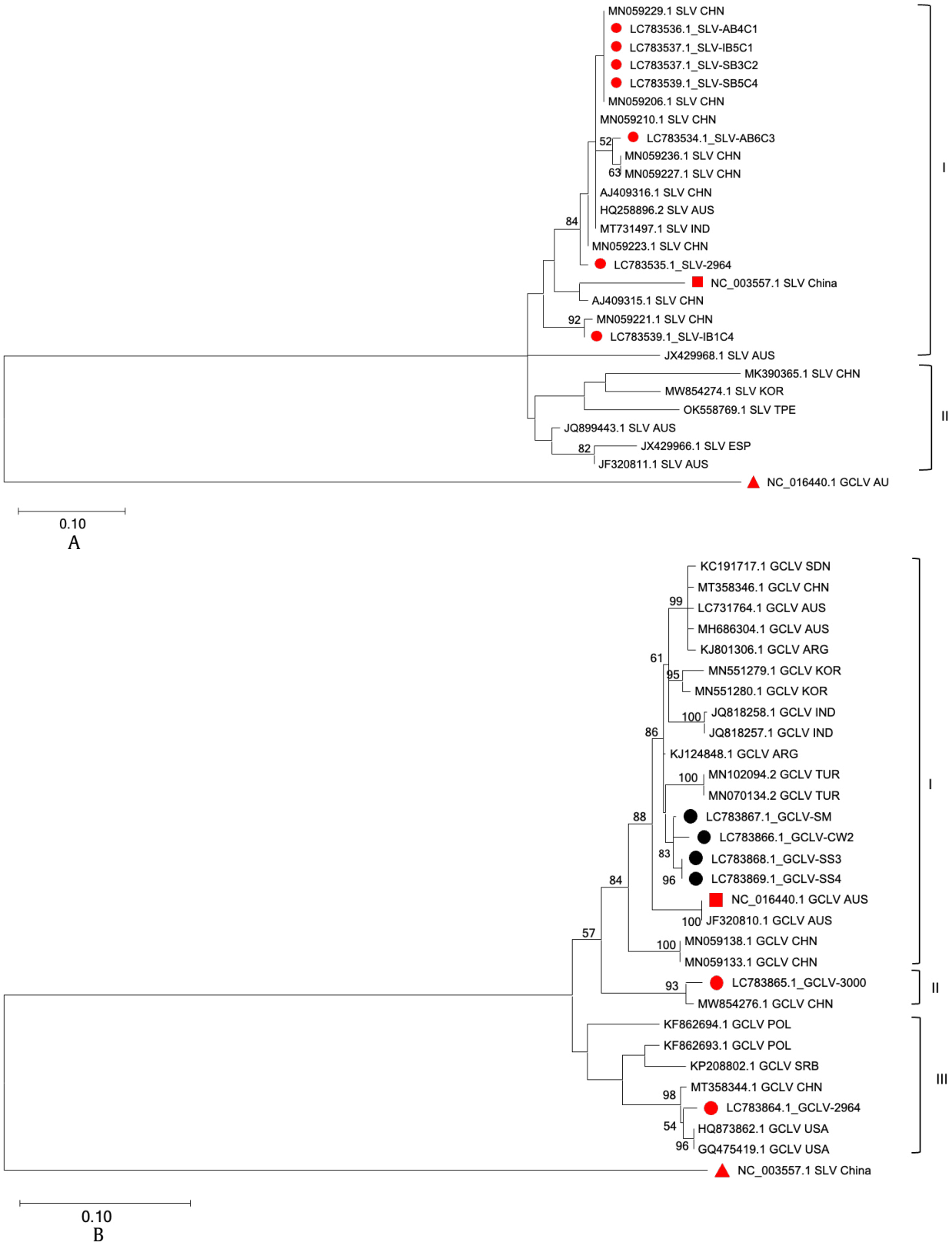

Figure 7. Maximum-likelihood phylogenetic trees of carlaviruses group infecting imported and Indonesian local garlic based on shallot latent virus (SLV) (A), garlic common latent virus (GCLV) (B). The phylogeny was implemented with MEGA X and bootstrap with 1000 replicates. The symbol ● designates the local garlic virus isolates (from seed bulbs), ● imported garlic virus isolates (from consumption bulbs), ■ the reference sequence, and ▲ the outgroup

bulbs from local garlic varieties. Although imported garlic is currently only used for consumption, it is still necessary to detect virus infection to anticipate the spread of the virus through imported garlic.

The data from this study shows that there are symptoms caused by virus infection in both seed and consumption bulbs. These symptoms can be seen on the leaves when garlic bulbs are grown. The symptoms involved leaf malformation, stunting, leaf curling, yellow mosaic, green mosaic, and leaf curling at the tip. Little is known about specific symptoms of virus infection in garlic bulbs. According to Conci *et al.* (2003) and Lunello *et al.* (2007), garlic bulbs infected with the virus experience a decrease in size and weight. Still, bulbs with larger sizes don't need to be virus-free bulbs, and smaller bulbs are bulbs that are infected with the virus. Cremer *et al.* (2021) stated that sometimes larger bulbs contain more virus titters than smaller bulbs. In addition, differences in bulb size can occur due to differences in varieties and environmental conditions when planting these bulbs. For example, the local seed bulbs in this study were smaller than the bulbs consumed by imports. This is because the two bulbs come from different varieties and different planting locations.

The results of virus detection using the RT-PCR method showed the distribution of viruses in several garlic production centers, especially in Central Java and West Nusa Tenggara (Figure 3, Table 2). OYDV and LYSV were found in all sampling locations, including Cianjur, West Java; Tawangmangu, Karanganyar, Central Java; and Sembalun, East Lombok, West Nusa Tenggara. In contrast, GCLV was only found in Cianjur, West Java, in 'Siam' and 'Ciwidey' and in Sembalun, East Lombok, and West Nusa Tenggara in 'Sangga Sembalun.' The distribution of garlic virus infection in Indonesia has been reported previously (Harti *et al.* 2020; Hidayat *et al.* 2023). According to Nurenik *et al.* (2021), garlic from Tawangmangu and Temanggung, Central Java, and garlic from Enrekang, South Sulawesi, were infected with OYDV and SLV, while those from Magelang, Central Java, were only infected with OYDV. In addition, OYDV, SLV, SYSV, and GCLV were also reported to have infected shallots in Brebes, Central Java; Probolinggo, East Java; Alahan Panjang, West Sumatra; Bima, West Nusa Tenggara; and Enrekang, South Sulawesi (Harti *et al.* 2020). Furthermore, mixed infections of both seed and consumption bulbs were confirmed.

The detection of the virus indicates that virus transmission can occur through bulbs (Figure 3). According to Parrano *et al.* (2012) and Mang *et al.* (2022), transmission of virus infection generally occurs due to the accumulation of virus particles in bulbs used as seeds in vegetative propagation. Although the bulbs are eventually used for consumption, the accumulation of virus particles can occur due to the use of infected bulbs in previous crops. This study showed that the consumption bulbs from the plant quarantine warehouse were infected by OYDV, LYSV, GCLV, and SLV (Table 2), but none of GCLV was found in the Bogor traditional market (Figure 3). Meanwhile, the results of previous research by Pauzi *et al.* (2018) showed that consumption bulbs from markets in the Bogor area were infected with GCLV and SLV. The bulbs had higher virus titters than 'Sangga Sembalun' as local seed bulbs. This indicates that consumption bulbs circulating in the community have been infected with several garlic viruses.

The transmission of viral infections in the fields can also occur through insect vectors. OYDV and LYSV are the most widespread garlic viruses in several countries, followed by GCLV and SLV (Mang *et al.* 2022). This study showed that OYDV and LYSV produced positive reactions in all test samples, both seed bulbs and consumption bulbs. OYDV and LYSV are also considered important viruses worldwide due to their wide distribution and have been widely reported to infect species of the Alliaceae family, such as garlic, shallots, and leeks (Barg *et al.* 1997; Harti *et al.* 2020; Mang *et al.* 2022). The wide distribution of OYDV and LYSV is one of the factors due to the non-persistent transmission of OYDV and LYSV through aphids in the field, which has a higher efficiency than GCLV and SLV (Gadhave *et al.* 2020; Yang *et al.* 2021; Mang *et al.* 2022). Virus-free garlic can be quickly re-infected after planting in the field, suggesting that the virus is efficiently transmitted by vectors, especially in adjacent infected plantings (Lot *et al.* 1998; Conci *et al.* 2003; Filho *et al.* 2006; Lunello *et al.* 2007).

Our study demonstrated the identification of OYDV, LYSV, and GCLV infecting both seed and consumption bulbs in Indonesia, except for SLV, which was only found in consumption bulbs. The result of this study also highlighted that the same species of viruses infecting both seed and consumption bulbs separated into different clades or types. According to the ICTV species demarcation for potyvirus and carlavirus, all samples in this study were still in their groups (Inoue-Nagata *et al.* 2022; Yoshikawa and Yaegashi 2021). A high-throughput sequencing (HTS) method

is needed to detect and anticipate the spread of new strains or new virus of garlic viruses in Indonesia. HTS is a new technology that is able to detect known and unknown plant viruses (Wylie *et al.* 2014). In addition, the elimination of viruses to provide seed health as planting material is expected to suppress the incidence of the virus in the field.

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