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*Research Article*

# Molecular diversity of citrus genotypes using *callose synthase 7* gene markers linked to Huanglongbing resistance

**Kristianto Nugroho [1,3], Agus Purwito [2*], Dewi Sukma [2], Mia Kosmiatin [3], Tri Joko Santoso [3], Ali Husni [4], Chaireni Martasari [3], and Puji Lestari [3]**

[1] Graduate Program of Plant Breeding and Biotechnology, Faculty of Agriculture, IPB University (Bogor Agricultural University), Jl. Meranti, Kampus IPB Dramaga, Bogor 16680, INDONESIA
[2] Department of Agronomy and Horticulture, Faculty of Agriculture, IPB University (Bogor Agricultural University), Jl. Meranti, Kampus IPB Dramaga, Bogor 16680, INDONESIA
[3] Research Center for Horticulture, Research Organization for Agriculture and Food, National Research and Innovation Agency, Jl. Raya Bogor Km. 46 Cibinong, Bogor 16911, INDONESIA
[4] Research Center for Animal Husbandry, Research Organization for Agriculture and Food, National Research and Innovation Agency, Jl. Raya Bogor Km. 46 Cibinong, Bogor 16911, INDONESIA
* Corresponding author (✉ apurwito@apps.ipb.ac.id)

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*IPB University*

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## ABSTRACT

*Huanglongbing (HLB), caused by Candidatus Liberibacter asiaticus, is a notable disease affecting citrus plantations globally. Several studies showed that the callose synthase 7 gene is crucial for the citrus defense system against this pathogen. The study aimed to analyze the nucleotide variations and genetic diversity among several citrus genotypes using specific gene primers designed from the callose synthase 7 gene sequence. Genomic DNA from eleven citrus genotypes was amplified using the specific primers, and Sanger sequencing was employed to identify the nucleotide sequence of the PCR products. The results revealed a total of 66 single-nucleotide polymorphisms (SNPs), 10 insertions, and 11 deletions were detected in callose synthase 7 gene fragment sequences. Of these, one out of five noteworthy SNPs identified at a position of 200 bp downstream of the START codon showed distinguishing features between susceptible and resistant/tolerant genotypes. Phylogenetic analysis clearly discriminated the eleven citrus genotypes into two clusters at a dissimilarity coefficient of 0.05, with all genotypes grouped in the first cluster, except for the Chinese box orange and orange jasmine. The identification of notable SNPs in this study can aid in developing new markers for the rapid selection of genotypes with enhanced HLB resistance in citrus breeding programs.*

**Keywords:** *Candidatus* Liberibacter asiaticus; nucleotide variations; Sanger sequencing; phylogenetic analysis; SNP

## INTRODUCTION

Huanglongbing (HLB), commonly known as citrus greening or citrus vein phloem degeneration (CVPD), is a significant disease affecting citrus plantations worldwide and has a destructive impact on citrus production. This disease is caused by a Gram-negative, uncultured bacterium that infects the phloem tissue of citrus plants (Jagoueix et al., 1994; Bové, 2006).

Three species of bacteria are responsible for causing diseases which led to significant yield losses in citrus plants: *Candidatus* Liberibacter asiaticus (*Ca.* L. asiaticus), which is prevalent in citrus plantations in Asia; *Candidatus* Liberibacter africanus (*Ca*. L. africanus), which affects citrus plantations in Africa; and *Candidatus* Liberibacter americanus (*Ca*. L. americanus), first identified in São Paulo, Brazil, and spreading throughout the America region (Jagoueix et al., 1994; Teixeira et al., 2005). *Candidatus* Liberibacter asiaticus is a heat-tolerant bacterium that is transmitted primarily by the Asian citrus psyllid (*Diaphorina citri*), which acts as a vector (Wang, 2019). Moreover, the bacteria could also be transmitted via grafting from diseased budwood to healthy rootstock (Albrecht et al., 2014).

The pathogen is responsible for several disease symptoms that are often mistaken for nutritional deficiencies in plants. These symptoms include chlorosis, blotchy mottling on leaves, stunted growth, leaf drop, fruit drop, a reduced number of fruits that are smaller in size, an unchanged green color at the stylar end, asymmetrical fruit locules, and bitter fruit with a metallic taste (da Graca et al., 2016; Dala-Paula et al., 2019; Shahzad et al., 2020; Tipu et al., 2021; Neupane et al., 2023). Since the early 1990s, this disease has devastated citrus plantations worldwide, causing severe economic and yield losses estimated in the millions of dollars (Nurhadi, 2015; Alvarez et al., 2016; Li et al., 2020).

Plant destruction occurs due to the deposition of a starch called callose in the phloem tissue of citrus plants. This initially serves as a defense response to reduce the movement of *Candidatus* Liberibacter asiaticus, but ultimately leads to phloem necrosis (Achor et al., 2010; Koh et al., 2012). Callose (β-1,3-glucan) is a complex carbohydrate that forms plant cell walls, alongside cellulose (β-1,4-glucan), hemicellulose, and pectin (Wang et al., 2022). In addition to its vital role in the cell wall complex, callose is essential for forming root hairs and sieve pores in the phloem, as well as in pollen and pollen tubes (Usak et al., 2023). The deposition of callose in phloem tissue plugs the sieve plates, impeding the flow of photosynthates from leaves to other plant organs, which inhibits plant growth activities (Etxeberria & Narciso, 2015).

Previous studies have shown that callose biosynthesis is regulated by a family of genes, specifically *callose synthase* (Verma & Hong, 2001). In *Arabidopsis thaliana*, 12 *callose synthase* genes have been identified, with only the *callose synthase 7* (*CalS7*) gene playing a specific role in callose deposition in phloem tissue (Pirselová & Matusikova, 2013). According to Peng et al. (2019) and Granato et al. (2019), a total of 12 *callose synthase* genes in citrus plants were identified, and these genes showed the evolutionary potential functional roles with those found in *A. thaliana*. Additionally, several studies have reported the identification of 32 *callose synthase* genes in canola (*Brassica napus*) (Liu et al., 2018), 27 genes in cotton (*Gossypium hirsutum*) (Feng et al., 2021), and 24 genes in soybean (*Glycine max*) (Zaynab et al., 2024).

The breeding program aimed at developing new and improved citrus varieties resistant to Huanglongbing is challenging due mainly to the limited availability of resistance donors, particularly from commonly cultivated citrus sources. It is widely known that most of the commercial citrus cultivars are susceptible to Huanglongbing disease (Bové, 2006). However, several citrus species and their related species have shown their ability to withstand the impact of this disease. Such citrus genotypes include orange jasmine (*Murraya paniculata*), khasi papeda (*Citrus latipes*), trifoliate orange (*Poncirus trifoliata*), Chinese box orange (*Severinia buxifolia*), Carrizo citrange (*Citrus sinensis* x *P. trifoliata*), Australian finger lime (*Microcitrus australasica*), US-897, Bearss lemon, LB8-9 Sugar Belle mandarin, and Bingo mandarin (Albrecht et al., 2016; Killiny et al., 2018; Rao et al., 2018; Deng et al., 2019). A study by Prasetyaningrum et al. (2012) found that several pomelo (*Citrus maxima*) cultivars, such as Pangkajene Putih, Magetan, Raja, and Pangkajene Merah, demonstrated their resistance to Huanglongbing, particularly when inoculated with the HLB pathogen. Additionally, Fan et al. (2012) reported that the rough lemon (*Citrus jambhiri*) genotype exhibited greater tolerance to Huanglongbing than sweet orange and other susceptible citrus varieties.

In plant breeding, having information related to the diversity of citrus germplasm is essential for guiding the focus of breeding programs. This study aimed to analyze the nucleotide variations and genetic diversity among several citrus genotypes using specific primers designed from the *CalS7* gene fragment sequence. The findings, particularly the nucleotide variations observed in the citrus genotypes, can promisingly aid in developing new molecular markers for the rapid selection of citrus germplasm resistant to Huanglongbing. This, in turn, will help accelerate the citrus breeding program.

## MATERIALS AND METHODS

### *Plant materials*

In this study, we meticulously analyzed a total of eleven distinct citrus genotypes (Table 1). These genetic materials were predominantly sourced from the Agency for Agricultural Assemblies and Modernization for Citrus and Subtropical Fruits (BRMP Jestro) in Malang, East Java, Indonesia.

On the other hand, the Chinese box-orange, orange jasmine, and Australian finger lime genotypes were generously provided by local farmers. Among the genotypes, Siam Pontianak, Siam Madu, Keprok Batu55, and Keprok Terigas represented commercially significant mandarin cultivars that are known to be susceptible to Huanglongbing disease. In contrast, Pangkajene Putih, Magetan, rough lemon, Carrizo citrange, orange jasmine, Chinese box orange, and Australian finger lime have demonstrated remarkable HLB resistance or tolerance genotypes, as evidenced by previous research findings (Folimonova et al., 2009; Prasetyaningrum et al., 2012; Fan et al., 2012; Ramadugu et al., 2016).

Table 1. Orange genotype and its source of planting material.

| No. | Genotypes name | Species | Source of collection |
|---|---|---|---|
| 1. | Siam Pontianak | *Citrus nobilis* L. | BRMP Jetsro |
| 2. | Siam Madu | *C. nobilis* L. | BRMP Jetsro |
| 3. | Keprok Batu55 | *Citrus reticulata* Blanco. | BRMP Jetsro |
| 4. | Keprok Terigas | *C. reticulata* Blanco. | BRMP Jetsro |
| 5. | Rough lemon | *C. jambhiri* Lush. | BRMP Jetsro |
| 6. | Pangkajene Putih | *C. maxima* Merr. | BRMP Jetsro |
| 7. | Magetan | *C. maxima* Merr. | BRMP Jetsro |
| 8. | Carrizo citrange | *C. sinensis* L. x *P. trifoliata* L. | BRMP Jetsro |
| 9. | Chinese box orange | *S. buxifolia* (Poiret) Tan. | Local farmer in Bandung, West Java |
| 10. | Australian finger lime | *M. australasica* (F. Muell.) Swing. | Local farmer in Batu, East Java |
| 11. | Orange jasmine | *M. paniculata* (L.) Jack. | Local farmer in Citayam-Bogor, West Java |

### *Designing specific gene primers*

A search for the sequence of the *CalS7* gene was employed using the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/). The mRNA sequence from NCBI was subsequently compared utilizing the Basic Local Alignment Search Tool nucleotide (BLASTn) program (Altschul et al., 1997) with data available on the Citrus Genome Database (https://www.citrusgenomedb.org/blast/nucleotide/nucleotide). The mRNA sequence obtained from NCBI was aligned with *Citrus sinensis* v1.0 genome (JGI) CDS in the Citrus Genome Database. The sequence was subsequently utilized for primer design via Primer3Plus (https://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi) (Untergasser et al., 2007). The primer sequences generated by Primer3Plus were subsequently validated using Primer-BLAST (https://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi) (Ye et al., 2012) to ensure that the primers possess a single annealing site. The sequences of the primers are displayed in Table 2.

Table 2. List of specific gene primers designed in this study.

| No. | Primers name | Sequences (5'-3') | Tm (°C) | Product size (bp) |
|---|---|---|---|---|
| 1. | CsCalS7-F | CAGATGTGGTTTATGGAATTTTG | 58.4 | 638 |
| 2. | CsCalS7-R | ATGATTCTTGGAAATCATTAAGCA | 59.1 | |

*Genomic DNA isolation, DNA amplification, and Sanger sequencing*

The adapted Doyle and Doyle (1990) method was employed to extract the genomic DNA prepared from citrus leaf samples. This process involved the addition of 2% (w/v) polyvinylpyrrolidone (PVP) and 0.38% (w/v) sodium bisulfite freshly to the extraction buffer. The resulting DNA solutions were adjusted to a final concentration of 20 ng $\mu L^{-1}$ and utilized as the working solution. DNA amplification for each sample was performed in a total volume of 40 µL, comprising 2 µL of 20 ng $\mu L^{-1}$ DNA template, 20 µL of 2×MyTaq™ HS Red Mix (Bioline, UK), 2 µL each of forward and reverse primers at a concentration of 10 µM, along with sterile $ddH_2O$.

The T100 Thermal Cycler (Bio-Rad, USA) machine was employed for the DNA amplification process under the following conditions: first denaturation for 5 min at 95 °C, followed by 35 cycles of denaturation for 30 s at 94 °C, annealing process for 1 min at 55 °C, and first extension for 1 min at 72 °C. The PCR reaction was concluded with the final extension step for 15 min at 60 °C. The PCR products were subsequently separated at 100 V for 25 min utilizing 1% (w/v) agarose gel in an electrophoresis containing 0.5× Tris Borate EDTA (TBE) buffer. The gel was then stained with 10 mg $mL^{-1}$ ethidium bromide and visualized using a UV transilluminator. Samples with clearly amplified bands were then sent to PT Genetika Science Indonesia for Sanger sequencing.

The Sanger sequencing results were further analyzed by Clustal Omega (https://www.ebi.ac.uk/jdispatcher/msa/clustalo) (Sievers & Higgins, 2014) to determine the nucleotide variations, specifically single-nucleotide polymorphisms (SNPs), insertions, and deletions. The phylogenetic tree was generated from the nucleotide sequences utilizing the unweighted pair group method with arithmetic (UPGMA) program and the Tamura-Nei model, incorporating 1000 bootstrap iterations in Mega X (Kumar et al., 2018).

## RESULTS AND DISCUSSION

*Design of specific gene primers*

Searching for the mRNA sequence of citrus *CalS7* in the NCBI database yielded the accession number XM_006484852.3. This accession represents the *Citrus sinensis* callose synthase 7-like gene (transcript variant X5) as predicted (LOC102620841). The nucleotide sequences exhibited 98.78% homology to a gene located at the orange1.1g022398m locus in the Citrus Genome Database. The gene sequence was 2,294 base pairs (bp) in length, with a coding sequence of 891 bp. The gene annotation indicates that the given gene is related to the 1,3-beta-glucan synthase component FKS1-like, domain-1, and it consists of seven exons interspersed with five introns. The forward primer was situated in the first exon, while the reverse primer was located in the third intron, as shown in Figure 1. These primers successfully amplified the region between the first and third exons, encompassing the first intron up to the early third intron.

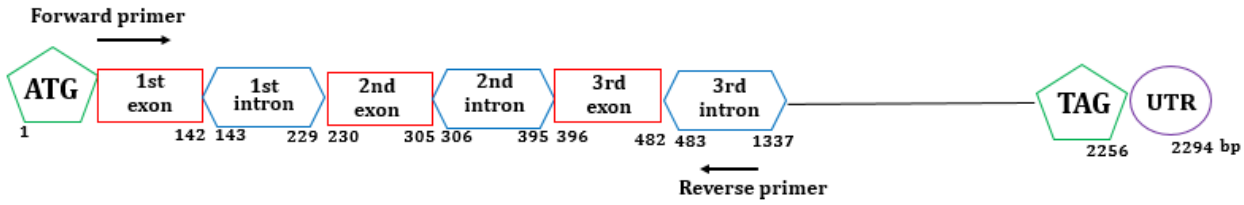


Figure 1. The forward and reverse primers' position in the citrus *callose synthase 7* gene sequence. Information: ATG: START codon, TAG: STOP codon, UTR: untranslated region.

The electrophoresis of agarose gel results indicated that the specific gene primers designed in this study could clearly amplify all of the citrus DNA, with distinct visualized amplicon bands (Figure 2). All of the PCR products showed a single amplicon band at the size of 638 bp, except for those derived from the orange jasmine genotype, showing a nonspecific band at approximately 900 bp in addition to the targeted band (Figure 2). Nonspecific amplicons might be influenced by various factors, including primer concentrations, annealing temperatures, the DNA template used, and *Taq* Polymerase slippage (Hosseinzadeh-Colagar et al., 2016; Ruiz-Villalba et al., 2017). Another possible reason for the nonspecific band could be the polymorphism present in the *CalS7* gene sequence of orange jasmine compared to the other citrus genotypes. Although orange jasmine is a relative of citrus from the Clauseneae tribe, it still belongs to the Rutaceae family (Tanruean et al., 2021). The utilization of primers that are specific to certain species, as demonstrated in this study, can create more polymorphism than using the general primers (Amiteye, 2021). However, the primer pairs are still selected based on how well they fit with research studies, even if they may not be the most effective or specific choice, as long as they can still create the desired amplicon band (Banos et al., 2018).

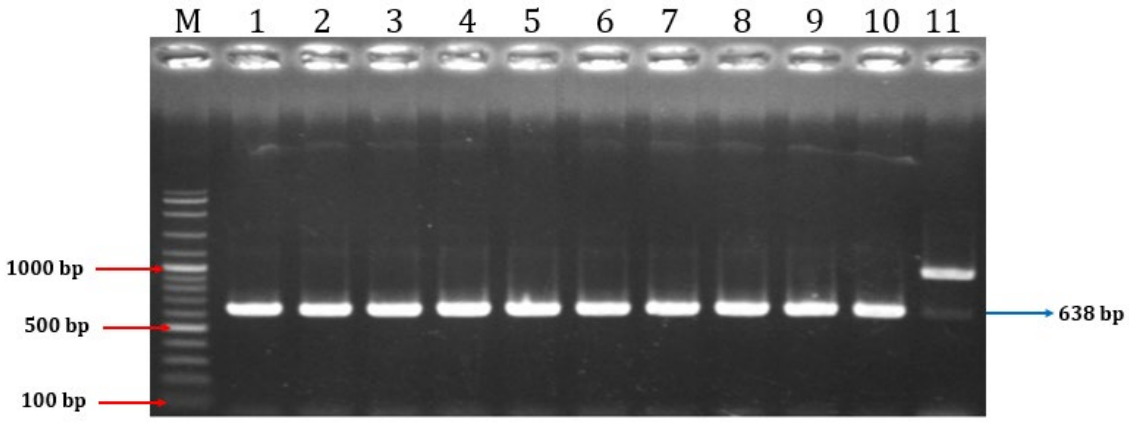


Figure 2. The banding pattern of PCR products from citrus genotypes that were amplified using *CalS7*-specific gene primers in 1% (w/v) agarose gel electrophoresis. Information: M: 100 bp plus DNA ladder (Vivantis Technologies), 1-11: citrus genotypes used in this study, with the order as presented in Table 1.

*Nucleotide variations among citrus genotypes*

A total of 87 nucleotide sequence variations in the *CalS7* gene fragment, including 66 (75.9%) SNPs, 10 (11.5%) insertions, and 11 (12.6%) deletions, were identified from all of the citrus genotypes (Figure 3). The majority of the nucleotide variations were categorized as SNPs, followed by deletions and insertions. Among the different genotypes analyzed, the orange jasmine cultivar exhibited the most nucleotide sequence variations, followed by the Chinese box orange.

This study discovered five noteworthy SNPs; however, only one SNP was found to effectively distinguish between susceptible and tolerant/resistant citrus genotypes (Table 3). Four significant SNPs at positions of 52, 381, 417, and 422 bp from the consensus sequence were detected in the genotypes Pangkajene Putih, Magetan, Carrizo citrange, Chinese box orange, and orange jasmine, but these SNPs were not found in Australian finger lime. Conversely, the SNP [G/A] was identified in Pangkajene Putih, Magetan, Carrizo citrange, Chinese box orange, orange jasmine, and Australian finger lime. This SNP was located at 145 bp from the consensus or 200 bp downstream of the start codon in the reference sequence.

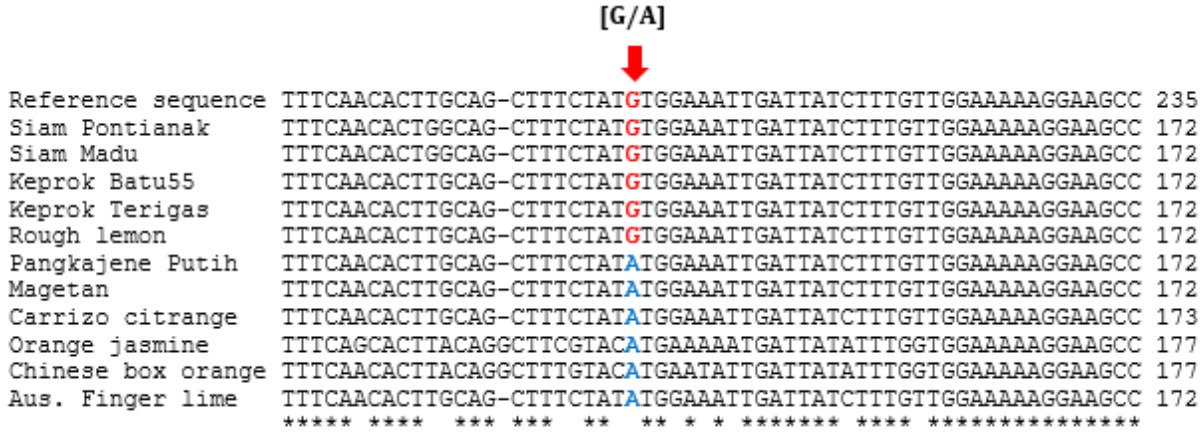


Figure 3. The SNP [G/A] at position 145 bp from the consensus or 200 bp downstream of the START codon at the reference sequence that distinguishes the susceptible from the resistant citrus genotypes against Huanglongbing disease.

All of the noteworthy SNPs detected in this study were not identified in rough lemon, even though a previous study from Fan et al. (2012) had reported that the rough lemon genotype showed good withstand to the Huanglongbing infection. On the contrary, Ramadugu et al. (2016) categorized rough lemon as predominantly susceptible to *Candidatus* Liberibacter asiaticus, displaying distinctive HLB symptoms based on field evaluations, supporting the results of our study. Additionally, our present study failed to identify any insertions or deletions that could differentiate between susceptible and tolerant/resistant genotypes.

Several studies have reported the discovery of SNPs that are related to disease resistance in plants. Cuenca et al. (2016) identified a biallelic SNP [G/T] in citrus, which is related to the *Alternaria* brown spot resistance gene. Xie et al. (2020) discovered the SNP in the *Pm5* locus that is related to common wheat's resistance to powdery mildew, while Liu et al. (2021) reported the SNP in the *GLUTAMATE RECEPTOR-LIKE* gene that is responsible for *Gossypium hirsutum* resistance to *Fusarium* wilt. The presence of SNPs, especially those located in a coding sequence with a possible change in the amino acid order (recognized as a non-synonymous SNP), could modify the protein's activity and also change the plant phenotype (Robert & Pelletier, 2018). Nevertheless, the SNP [G/A] that could distinguish among susceptible and tolerant/resistant genotypes in our study was found in the first intron area. Previous study from Koeda et al. (2021) also reported the discovery of the SNP [A/G] in the intron of the *pepy-1* gene that could distinguish between the resistance and susceptible chili pepper genotypes to Pepper Yellowing Leaf Curl Disease (PepYLCD). However, the presence of a SNP in the intron area can still be potentially useful as a selection marker in a breeding program.

Table 3. List of noteworthy SNPs identified in this study.

| Position from consensus (bp) | 52 | 145 | 381 | 417 | 422 |
|---|---|---|---|---|---|
| Position from START codon in reference sequence (bp) | 114 | 200 | 434 | 470 | 475 |
| Reference sequence | C | G | C | T | T |
| Siam Pontianak | C | G | C | T | T |
| Siam Madu | C | G | C | T | T |
| Keprok Batu55 | C | G | C | T | T |
| Keprok Terigas | C | G | C | T | T |
| Rough lemon | C | G | C | T | T |
| Pangkajene Putih | T | A | T | C | G |
| Magetan | T | A | T | C | G |
| Carrizo citrange | T | A | T | C | G |
| Chinese box orange | T | A | T | C | G |
| Orange jasmine | T | A | T | C | G |
| Australian finger lime | C | A | C | T | T |
| SNP location | First exon | First intron | Third exon | Third exon | Third exon |

*Phylogenetic analysis of citrus genotypes*

The phylogenetic analysis based on the *CalS7* gene fragment showed a clear separation among eleven citrus genotypes into two clusters at a dissimilarity coefficient of 0.05 (Figure 4). All of the genotypes were grouped in the first cluster, except for Chinese box orange and orange jasmine, which were both separated in the second cluster. This clustering pattern aligned with the previous results that the most nucleotide sequence variations were detected in the orange jasmine and the Chinese box orange. The pairwise distance showed the highest sequence similarity obtained between Keprok Batu55 and Siam Pontianak, as well as between Keprok Terigas and Siam Madu, with a similarity of 99.80% (Table 4). The lowest sequence similarity of 88.80% was obtained between Carrizo citrange and Chinese box orange The finding of a notable SNP, which was detected in the resistance/tolerance citrus genotypes, could provide useful information in designing a functional markers, such as single nucleotide amplified polymorphism (SNAP) marker which can potentially be used as a rapid screening tool for citrus germplasm resistant to Huanglongbing disease in the future. Several studies have demonstrated the utilization of SNAP markers in discriminating the plant genotypes, such as SNAP markers associated with disease resistance genes in banana by Sutanto et al. (2013), *Phalaenopsis* orchid by Sukma et al. (2021), and cacao by Tarigan et al. (2021).

According to Pirselova´and Matusikova (2013), the *CalS7* gene exhibited a specific role in callose deposition in phloem tissue. A study from Granato et al. (2019) showed the increase in *callose synthase 2* (*CalS2*) and *CalS7* gene activities in Huanglongbing-inoculated citrus plants at 120 days post-inoculation based on real-time PCR, and also the increase in *CalS7* and *callose synthase 12* (*CalS12*) gene activities at 360 days post-inoculation. The results presented the essential role of *CalS7* gene in citrus defense systems against Huanglongbing infection. In addition, Xie et al. (2021) reported the increased activities of *starch synthase* and *callose synthase* genes after Huanglongbing infection. Bernardini et al. (2024) also reported the increase of callose concentrations in roots, leaves, midribs, stems, and peduncles of Huanglongbing-infected citrus plants compared to healthy plants.

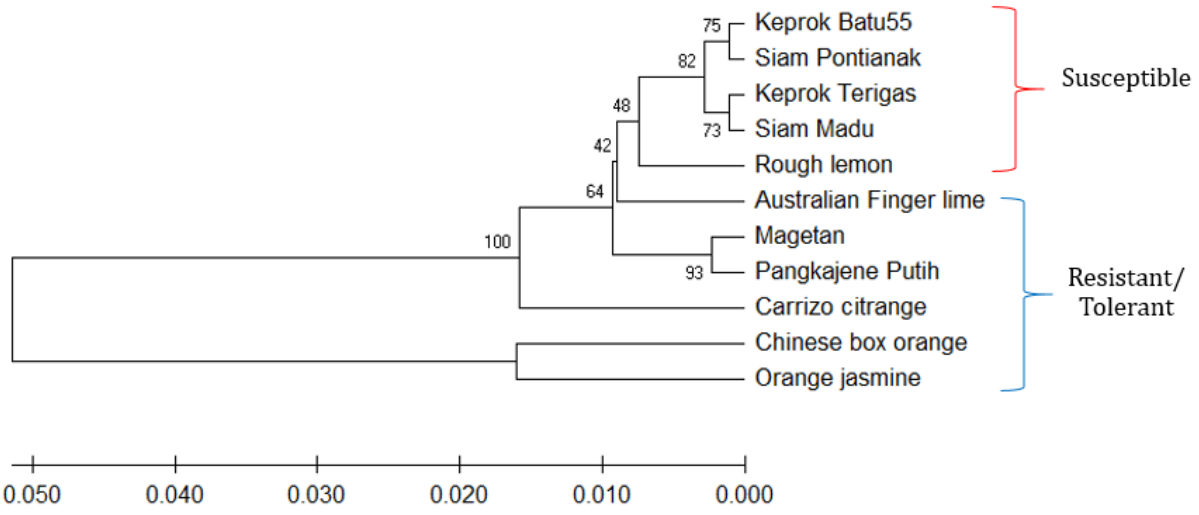


Figure 4. Phylogenetic tree of citrus genotypes used in this study, based on nucleotide variations in *CalS7* gene fragment, constructed using the UPGMA method.

Table 4. Pairwise distance among citrus genotypes used in this study based on *CalS7* gene fragment.

| Genotypes | Carrizo citrange | Chinese box orange | Australian finger lime | Orange jasmine | Keprok Batu55 | Keprok Terigas | Magetan | Pangka jene Putih | Rough lemon | Siam Madu |
|---|---|---|---|---|---|---|---|---|---|---|
| Carrizo citrange | | | | | | | | | | |
| Chinese box orange | 0.112 | | | | | | | | | |
| Australian finger lime | 0.035 | 0.110 | | | | | | | | |
| Orange jasmine | 0.110 | 0.032 | 0.105 | | | | | | | |
| Keprok Batu55 | 0.035 | 0.107 | 0.018 | 0.105 | | | | | | |
| Keprok Terigas | 0.030 | 0.102 | 0.014 | 0.100 | 0.004 | | | | | |
| Magetan | 0.020 | 0.092 | 0.018 | 0.090 | 0.018 | 0.014 | | | | |
| Pangkajene Putih | 0.023 | 0.092 | 0.023 | 0.090 | 0.018 | 0.014 | 0.005 | | | |
| Rough lemon | 0.042 | 0.107 | 0.021 | 0.105 | 0.016 | 0.011 | 0.021 | 0.025 | | |
| Siam Madu | 0.032 | 0.104 | 0.016 | 0.103 | 0.007 | 0.002 | 0.016 | 0.016 | 0.014 | |
| Siam Pontianak | 0.037 | 0.110 | 0.021 | 0.108 | 0.002 | 0.007 | 0.021 | 0.021 | 0.018 | 0.004 |

The discovery of significant SNPs in the *CalS7* gene fragment, which can differentiate between the susceptible and tolerant/resistant citrus genotypes, is reinforced by the clustering pattern observed in the phylogenetic tree. This finding adds valuable information to the ongoing research on citrus resistance against Huanglongbing disease. Future studies should be further carried out to comprehensively investigate this gene, particularly in elucidating its role and functions during Huanglongbing infection in both susceptible and resistant genotypes. Additionally, this gene may serve as a target for genome editing research to understand its impact on the citrus defense system against Huanglongbing infection. This information is expected to contribute to the enhancement

of the citrus breeding program, which specifically aimed at tackling the Huanglongbing issue in citrus plantations, as noted by Killiny et al. (2018), that all roads lead to Rome to overcome the Huanglongbing disease.

## CONCLUSIONS

The analysis of genetic diversity in eleven citrus genotypes based on *callose synthase 7* gene fragment sequence presented the nucleotide sequence variations among those genotypes. Most of the nucleotide variations detected in respective genes were SNPs, followed by deletions and insertions. There were five noteworthy SNPs detected, but only one SNP [G/A] at the position of 200 bp downstream of the START codon showed the ability to distinguish between susceptible and tolerance/resistance citrus genotypes. The phylogenetic analysis showed the distinct separation among citrus genotypes in two main clusters, with the main distinction being presented by Chinese box orange and orange jasmine genotypes. The identification of these key SNPs enhances our understanding of the genetic diversity and offers promising implications in developing more resilient citrus varieties, thereby supporting agricultural advancement and food security.

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## REFERENCES

Achor, D. S., Etxeberria, E., Wang, N., Folimonova, S. Y., Chung, K. R., & Albrigo, L. G. (2010). Sequence of anatomoical symptom observations in citrus affected with Huanglongbing disease. *Plant Pathology Journal*, *9*, 56–64. https://doi.org/10.3923/ppj.2010.56.64

Albrecht, U., Fiehn, O., & Bowman, K. D. (2016). Metabolic variations in different citrus rootstock cultivars associated with different responses to Huanglongbing. *Plant Physiology and Biochemistry*, *107*, 33-44. https://doi.org/10.1016/j.plaphy.2016.05.030

Albrecht, U., Hall, D. G., & Bowman, K. D. (2014). Transmission efficiency of *Candidatus* Liberibacter asiaticus and progression of Huanglongbing disease in graft-and psyllid-inoculated citrus. *HortScience*, *49*(3), 367-377. https://doi.org/10.21273/HORTSCI.49.3.367

Altschul, S. F., Gish, W., Miller, W., Myers, E. W., & Lipman, D. J. (1990). Basic local alignment search tool. *Journal of Molecular Biology*, *215*(3), 403-410. https://doi.org/10.1016/S0022-2836(05)80360-2

Alvarez, S., Rohrig, E., Solís, D., & Thomas, M. H. (2016). Citrus greening disease (Huanglongbing) in Florida: economic impact, management and the potential for biological control. *Agricultural Research*, *5*, 109-118. https://doi.org/10.1007/s40003-016-0204-z

Amiteye, S. (2021). Basic concepts and methodologies of DNA marker systems in plant molecular breeding. *Heliyon*, *7*(10), e08093. https://doi.org/10.1016/j.heliyon.2021.e08093

Banos, S., Lentendu, G., Kopf, A., Wubet, T., Glöckner, F. O., & Reich, M. (2018). A comprehensive fungi-specific 18S rRNA gene sequence primer toolkit suited for diverse research issues and sequencing platforms. *BMC Microbiology*, *18*, 190. https://doi.org/10.1186/s12866-018-1331-4

Bernardini, C., Franco, A., Russo, R., Livingston, T., Gmitter Jr., F. G., Levy, A., & Vashisth, T. (2024). An efficient method for the extraction and the quantitative determination of callose from HLB-affected and healthy citrus. *Frontiers in Agronomy*, *6*, 1307566. https://doi.org/10.3389/fagro.2024.1307566

Bové, J. M. (2006). Huanglongbing: a destructive, newly-emerging, century-old disease of citrus. *Journal of Plant Pathology*, *88*, 7–37.

Cuenca, J., Aleza, P., Garcia-Lor, A., Ollitrault, P., & Navarro, L. (2016). Fine mapping for identification of citrus alternaria brown spot candidate resistance genes and development of new SNP markers for marker-assisted selection. *Frontiers in Plant Science*, 7, 1948. https://doi.org/10.3389/fpls.2016.01948

da Graça, J. V., Douhan, G. W., Halbert, S. E., Keremane, M. L., Lee, R. F., Vidalakis, G., & Zhao, H. (2016). Huanglongbing: an overview of a complex pathosystem ravaging the world's citrus. *Journal of Integrative Plant Biology*, *58*(4), 373-387. https://doi.org/10.1111/jipb.12437

Dala-Paula, B. M., Plotto, A., Bai, J., Manthey, J. A., Baldwin, E. A., Ferrarezi, R. S., & Gloria, M. B. A. (2019). Effect of Huanglongbing or greening disease on orange juice quality, a review. *Frontiers in Plant Science*, *9*, 1976. https://doi.org/10.3389/fpls.2018.01976

Deng, H., Achor, D., Exteberria, E., Yu, Q., Du, D., Stanton, D., Liang, G., & Gmitter Jr., F. G. (2019). Phloem regeneration is a mechanism for Huanglongbing-tolerance of "Bearss" lemon and "LB8-9" Sugar Belle® mandarin. *Frontiers in Plant Science*, *10*, 277. https://doi.org/10.3389/fpls.2019.00277

Doyle, J. J., & Doyle, J. L. (1990). Isolation of plant DNA from fresh tissue. *Focus*, *12*(1), 13-15.

Etxeberria, E., & Narciso, C. (2015). Anatomy of the citrus leaf petiole: healthy vs. Huanglongbing. *Acta Horticulturae*, *1065*, 891–897. https://doi.org/10.17660/ActaHortic.2015.1065.110

Fan, J., Chen, C., Yu, Q., Khalaf, A., Achor, D. S., Brlansky, R. H., Moore, G. A., Li, Z. G., & Gmitter Jr., F. G. (2012). Comparative transcriptional and anatomical analyses of tolerant rough lemon and susceptible sweet orange in response to '*Candidatus* Liberibacter asiaticus' infection. *Molecular Plant-Microbe Interaction*, *25*(11), 1396-1407. http://dx.doi.org/10.1094/MPMI-06-12-0150-R

Feng, J., Chen, Y., Xiao, X., Qu, Y., Li, P., Lu, Q., & Huang, J. (2021). Genome-wide analysis of the *CalS* gene family in cotton reveals their potential roles in fiber development and responses to stress. *PeerJ*, *9*, e12557. https://doi.org/10.7717/peerj.12557

Folimonova, S. Y., Robertson, C. J., Garnsey, S. M., Gowda, S., & Dawson, W. O. (2009). Examination of the responses of different genotypes of citrus to Huanglongbing (citrus greening) under different conditions. *Phytopathology, 99*(12), 1346-1354. https://doi.org/10.1094/PHYTO-99-12-1346

Granato, L. M, Galdeano, D. M., D'Alessandre, N. D. R., Breton, M. C., & Machado, M. A. (2019). *Callose synthase* family genes plays an important role in the citrus defense response to *Candidatus* Liberibacter asiaticus. *European Journal of Plant Pathology*, *155*, 25-38. https://doi.org/10.1007/s10658-019-01747-6

Hosseinzadeh-Colagar, A., Haghighatnia, M. J., Amiri, Z., Mohadjerani, M., & Tafrihi, M. (2016). Microsatellite (SSR) amplification by PCR usually led to polymorphic bands: evidence which shows replication slippage occurs in extend or nascent DNA strands. *Molecular Biology Research Communications*, *5*(3), 167-174.

Jagoueix, S., Bové, J. M., & Garnier, M. (1994). The phloem-limited bacterium of greening disease of citrus is a member of the α subdivision of the Proteobacteria. *International Journal of Systematic Bacteriology*, *44*(3), 379–386. https://doi.org/10.1099/00207713-44-3-379

Killiny, N., Jones, S. E., Nehela, Y., Hijaz, F., Dutt, M., Gmitter, F. G, Grosser, J. W. (2018). All roads lead to Rome: towards understanding different avenues of tolerance to Huanglongbing in citrus cultivars. *Plant Physiology and Biochemistry*, *129*, 1-10. https://doi.org/10.1016/j.plaphy.2018.05.005

Koeda, S., Onouchi, M., Mori, N., Pohan, N. S., Nagano, A. J., & Kesumawati, E. (2021). A recessive gene *pepy-1* encoding Pelota confers resistance to Begomovirus isolates of PepYLCIV and PepYLCAV in *Capsicum annuum*. *Theoretical and Applied Genetics*, *134*(9), 2947-2964. https://doi.org/10.1007/s00122-021-03870-7

Koh, E. J., Zhou, L., Williams, D. S., Park, J., Ding, N., Duan, Y. P., & Kang, B. H. (2012). Callose deposition in the phloem plasmodesmata and inhibition of phloem transport in citrus leaves infected with "*Candidatus* Liberibacter asiaticus". *Protoplasma*, *249*, 687-697. https://doi.org/10.1007/s00709-011-0312-3

Kumar, S., Stecher, G., Li, M., Knyaz, C., & Tamura. K. (2018). MEGA X: molecular evolutionary genetics analysis across computing platforms. *Molecular Biology and Evolution*, *35*(6), 1547–1549. doi: https://doi.org/10.1093/molbev/msy096

Li, S., Wu, F., Duan, Y., Singerman, A., & Guan, Z. (2020). Citrus greening: management strategies and their economic impact. *HortScience*, *55*(5), 604–612. https://doi.org/10.21273/HORTSCI14696-19

Liu, F., Zou, Z., & Fernando, W. G. D. (2018). Characterisation of callose deposition and analysis of the *callose synthase* gene family of *Brassica napus* in response to *Leptosphaeria maculans*. *International Journal of Molecular Science*, *19*(12), 3796. https://doi.org/10.3390/ijms19123769

Liu, S., Zhang, X., Xiao, S., Ma, J., Shi, W., Qin, T., Xi, H., Nie, X., You, C., Xu, Z., Wang, T., Wang, Y., Zhang, Z., Li, J., Kong, J., Aierxi, A., Yu, Y., Lindsey, K., Klosterman, S. J., Zhang, X., & Zhu, L. (2021). A single-nucleotide mutation in a *GLUTAMATE RECEPTOR-LIKE* gene confers resistance to *Fusarium* wilt in *Gossypium hirsutum*. *Advanced Science*, *8*(7), 2002723. https://doi.org/10.1002/advs.202002723

Neupane, A., Shahzad, F., Bernardini, C., Levy, A., & Vashisth, T. (2023). Poor shoot and leaf growth in Huanglongbing-affected sweet orange is associated with increased investment in defenses. *Frontiers in Plant Science*, *14*, 1305815. https://doi.org/10.3389/fpls.2023.1305815

Nurhadi, N. 2015. Huanglongbing disease (*Candidatus* Liberibacter asiaticus) on citrus: threats and control strategy. (In Indonesian.). *Pengembangan Inovasi Pertanian*, *8*(1), 21–32

Peng, Y., Fan, H., Lei, T., He, Y., Chen, S., & Yao, L. (2019). Expression analysis of callose synthase gene family in citrus. *Acta Horticulturae Sinica*, *46*(2): 330-336

Pirselova, B., & Matusikova, I. (2013). Callose: the plant cell wall polysaccharide with multiple biological functions. *Acta Physiologiae Plantarum*, *35*, 635–644. https://doi.org/10.1007/s11738-012-1103-y

Prasetyaningrum, P., Purwantoro, A., & Subandiyah, S. (2012). Genetics diversity analysis of six difference varieties of pomelo (*Citrus maxima* (Burm.)Merr.) for resistance to Huanglongbing using RAPD markers. *Vegetalika*, *1*(2), 78-85. https://doi.org/10.22146/veg.1521

Ramadugu, C., Keremane, M. L., Halbert, S. E., Duan, Y. P., Roose, M. L., Stover, E., & Lee, R. F. (2016). Long-term field evaluation reveals Huanglongbing resistance in citrus relatives. *Plant Disease*, *100*(9), 1858-1869. https://doi.org/10.1094/PDIS-03-16-0271-RE

Rao, M. J., Ding, F., Wang, N., Deng, X., & Xu, Q. (2018). Metabolic mechanisms of host species against citrus Huanglongbing (greening disease). *Critical Reviews in Plant Sciences*, *37*(6), 496-511. https://doi.org/10.1080/07352689.2018.1544843

Robert, F., & Pelletier, J. (2018). Exploring the impact of single-nucleotide polymorphisms on translation. *Frontiers in Genetics*, *9*, 507. https://doi.org/10.3389/fgene.2018.00507

Ruiz-Villalba, A., van Pelt-Verkuil, E., Gunst, Q. D., Ruijter, J. M., & van den Hoff, M. J. B. (2017). Amplification of nonspecific products in quantitative polymerase chain reactions (qPCR). *Biomolecular Detection and Quantification*, *14*, 7-18. https://doi.org/10.1016/j.bdq.2017.10.001

Shahzad, F., Chun, C., Schumann, A., & Vashisth, T. (2020). Nutrient uptake in huanglongbing-affected sweet orange: transcriptomic and physiological analysis. *Journal of the American Society for Horticultural Science*, *145*(6), 349–362. https://doi.org/10.21273/JASHS04929-20

Sievers, F., & Higgins, D. G. (2014). Clustal omega. *Current Protocols in Bioinformatics*, *48*(1), 3.13.1-3.13.16. https://doi.org/10.1002/0471250953.bi0313s48

Sukma, D., Elina, J., Raynalta, E., Aisyah, S. I., Aziz, S. A., Sudarsono, S., & Chan, M. T. (2021). Analysis of the genetic diversity of *Phalaenopsis* orchids with single nucleotide polymorphisms and SNAP markers derived from the *Pto* gene. *SABRAO Journal of Breeding and Genetics*, *53*(4), 620-631. https://doi.org/10.54910/sabrao2021.53.4.6

Sutanto, A., Hermanto, C., Sukma, D., & Sudarsono, S. (2013). Development of SNAP marker based on resistance gene analogue genomic sequences in banana (*Musa* spp.). (In Indonesian.). *Jurnal Hortikultura*, *23*(4), 300-309.

Tanruean, K., Poolprasert, P., Suwannarach, N., Kumla, J., & Lumyong, S. (2021). Phytochemical analysis and evaluation of antioxidant and biological activities of extracts from three clauseneae plants in Northern Thailand. *Plants*, *10*(1), 117. https://doi.org/10.3390/plants10010117

Tarigan, R., Maharijaya, A., & Izzah, N. K. (2021). SNAP markers derived from *catalase-1* gene sequence used for black pod disease resistance in cacao (*Theobroma cacao* L.). *SABRAO Journal of Breeding and Genetics*, *53*(3): 510-526.

Teixeira, D. C., Danet, J. L., Eveillard, S., Martins, E. C., Jesus Jr., W. C., Yamamoto, P. T., Lopes, S. A., Bassanezi, E. B., Ayres, A. J., Saillard, C., & Bové, J. M. (2005). Citrus Huanglongbing in São Paulo, Brazil: PCR detection of the '*Candidatus* Liberibacter' species associated with the disease. *Molecular and Cellular Probes*, *19*, 173–179. https://doi.org/10.1016/j.mcp.2004.11.002

Tipu, M. M. H., Masud, M. M., Jahan, R., Baroi, A., & Hoque, A. K. M. A. (2021). Identification of citrus greening based on visual symptoms: a grower's diagnostic toolkit. *Heliyon*, *7*(11), e08387. https://doi.org/10.1016/j.heliyon.2021.e08387

Untergasser, A., Nijveen, H., Rao, X., Bisseling, T., Geurts, R., & Leunissen, J. A. M. (2007). Primer3Plus, an enhanced web interface to Primer3. *Nucleic Acids Research*, *35*(2), 71–74. https://doi.org/10.1093/nar/gkm306

Usak, D., Haluska, S., & Pleskot, R. (2023). Callose synthesis at the center point of plant development—an evolutionary insight. *Plant Physiology*, *193*(1), 54–69. https://doi.org/10.1093/plphys/kiad274

Verma, D., & Hong, Z. (2011). Plant callose synthase complexes. *Plant Molecular Biology*, *47*, 693-701. https://doi.org/10.1023/a:1013679111111

Wang, B., Andargie, M., & Fang, R. (2022). The function and biosynthesis of callose in high plants. *Heliyon*, *8*(4), e09248. https://doi.org/10.1016/j.heliyon.2022.e09248

Wang, N. (2019). The citrus Huanglongbing crisis and potential solutions. *Molecular Plant*, *12*(5), 607–609. https://doi.org/10.1016/j.molp.2019.03.008

Xie, J., Guo, G., Wang, Y., Hu, T., Wang, L., Li, J., Qiu, D., Li, Y., Wu, Q., Lu, P., Chen, Y., Dong, L., Li, M., Zhang, H., Zhang, P., Zhu, K., Li, B., Deal, K. R., Huo, N., Zhang, Y., Luo, M. C., Liu, S., Gu, Y. Q., Li, H., & Liu, Z. (2020). A rare single nucleotide variant in *Pm5e* confers powdery mildew resistance in common wheat. *New Phytologist*, *228*(3), 1011-1026. https://doi.org/10.1111/nph.16762

Xie, Z., Zhao, K., Long, J., Zhen, L., Zou, X., Chen, S. (2021). Comparative analysis of Wanjincheng orange leaf and rootresponses to '*Candidatus* liberibacter asiaticus' infection using leaf-disc grafting. *Horticultural Plant Journal*, *7*(5), 401–410. https://doi.org/10.1016/j.hpj.2021.01.007

Ye, J., Coulouris, G., Zaretskaya, I., Cutcutache, I., Rozen, S., & Madden, T. (2012). Primer-BLAST: a tool to design target-specific primers for polymerase chain reaction. *BMC Bioinformatics, 13*, 134. https://doi.org/10.1186/1471-2105-13-134

Zaynab, M., Xu, Z. S., Yad, H. A., Hussain, A., Sharif, Y., Al-Yahyai, R., Sadder, M., Aloufi, A. S., & Li, S. (2024). Genome-wide analysis and expression profiling of *CalS* genes in *Glycine max* revealed their role in development and salt stress. *Journal of King Saud University – Science*, *36*, 103049. https://doi.org/10.1016/j.jksus.2023.103049