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Detection and Molecular Identification of *Candidatus Liberibacter asiaticus* in Mandarin Citrus (*Citrus reticulata*), a Local Cultivar from Tejakula, Bali, Indonesia, Based on 16S rRNA Gene Analysis

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ABSTRACT

Citrus Vein Phloem Degeneration (CVPD), also known as Huanglongbing (HLB), has become one of the main challenges in citrus production in Indonesia, including mandarin citrus var. Tejakula from Bali. The disease is associated with *Candidatus Liberibacter asiaticus* (CLas), a bacterium that inhabits phloem tissue and cannot be maintained in artificial culture, making molecular approaches essential for its detection. This study aimed to confirm the presence of CLas in mandarin citrus var. Tejakula in Bali using 16S rRNA gene analysis. Leaf samples showing CVPD-like symptoms were collected from four different locations and subjected to DNA extraction. Detection was performed using PCR with specific primers, followed by electrophoresis and sequence analysis. The results indicated that two samples, originating from Pecatu and Tukad Sumaga, were positively detected. Sequence comparison revealed a high level of similarity with reference sequences available in GenBank, confirming the identity of the pathogen. These findings provide molecular evidence of CLas infection in Bali and demonstrate the reliability of 16S rRNA-based detection for CVPD diagnosis.

Keywords: Candidatus Liberibacter asiaticus, CVPD, mandarin citrus, Tejakula, 16S rRNA

INTRODUCTION

Mandarin citrus var. Tejakula (*Citrus reticulata* var. Tejakula) represents one of the important local citrus commodities cultivated in northern Bali. This cultivar has been officially recognized as a superior national variety due to its economic value, distinctive fruit characteristics, and its wide cultivation across the region. Despite its potential, the sustainability of Tejakula mandarin production has been increasingly threatened by the presence of Citrus Vein Phloem Degeneration (CVPD), also widely known as Huanglongbing (HLB).

Globally, HLB is considered one of the most destructive diseases affecting citrus production, causing severe yield losses and decline in tree health in major citrus-growing regions. The disease has been reported extensively across Asia, Africa, and the Americas, highlighting its wide geographical distribution and significant economic impact. In Indonesia, CVPD continues to be a major

limiting factor in citrus cultivation, with Bali recognized as one of the endemic areas. Reports have indicated that a large proportion of mandarin plantations in northern Bali have been affected, resulting in decreased productivity and, in many cases, the removal of infected trees. The spread of this disease is closely related to the use of infected planting materials and the activity of insect vectors, particularly *Diaphorina citri*, emphasizing the importance of accurate pathogen detection.

The occurrence of CVPD is strongly associated with *Candidatus Liberibacter asiaticus* (CLas), a Gram-negative bacterium that resides in the phloem tissue of infected plants. Infection by this bacterium disrupts nutrient transport within the plant, leading to characteristic symptoms such as leaf chlorosis with green veins, stunted growth, malformed fruits, and eventual plant decline. One of the main challenges in studying CLas is its inability to be cultured under standard laboratory conditions, which limits the use of conventional microbiological techniques and necessitates the application of molecular-based detection methods.

Among the available molecular approaches, amplification of the 16S rRNA gene has been widely utilized for bacterial identification and classification. This gene contains both conserved and variable regions, allowing reliable detection as well as taxonomic differentiation of bacterial species. In the context of CLas detection, 16S rRNA-based PCR assays have been extensively applied due to their practicality and effectiveness. In addition, sequencing of amplified fragments enables further confirmation through comparison with reference sequences and supports phylogenetic analysis to understand genetic relationships among isolates.

Although CVPD has been widely reported in Bali, studies providing molecular confirmation of CLas infection in mandarin citrus var. Tejakula using sequence-based approaches are still limited. Most previous reports have focused primarily on disease incidence and field observations, while fewer have integrated PCR detection with sequencing analysis to validate pathogen identity. Therefore, this study was conducted to detect and molecularly identify *Candidatus Liberibacter asiaticus* infecting mandarin citrus var. Tejakula in Bali using 16S rRNA gene analysis.

MATERIALS AND METHODS

Study Area and Sample Collection

This study was conducted from July to December 2025. Leaf samples were collected from mandarin citrus var. Tejakula (*Citrus reticulata* var. *Tejakula*) plants showing typical Citrus Vein Phloem Degeneration (CVPD) symptoms, including leaf chlorosis with green veins, vein corking, and stunted growth (Figure 1). Sampling was carried out in four mandarin citrus cultivation areas in Bali, namely Tejakula Village (Buleleng Regency), Tukad Sumaga Village (Buleleng Regency), Pecatu Village (Badung Regency), and Balai Benih Induk Luwus (Tabanan Regency). Symptomatic leaves were collected using purposive sampling and stored in sterile plastic bags prior to molecular analysis.



Figure 1. Typical symptoms of Citrus Vein Phloem Degeneration (CVPD) observed on mandarin citrus var. *Tejakula*, showing leaf chlorosis with green veins

DNA Extraction

Total genomic DNA was extracted from fresh leaf midrib tissues using a Genomic DNA Mini Kit for Plants (Geneaid Biotech Ltd., Taiwan), following the manufacturer's instructions. Approximately 50–100 mg of leaf tissue was ground in liquid nitrogen to obtain a fine powder. The powdered tissue was mixed with lysis buffer and RNase A, followed by incubation at 60 °C for 10 minutes. Subsequent steps included precipitation, filtration, washing, and elution processes as described in the kit protocol. DNA concentration was measured using a NanoDrop spectrophotometer (Thermo Scientific, USA). The purified DNA was eluted in 100 µL of elution buffer and stored at −20 °C until PCR analysis.

PCR Amplification of 16S rRNA Gene

Detection of *Candidatus Liberibacter asiaticus* was performed using polymerase chain reaction (PCR) targeting the 16S rRNA gene. Amplification was carried out using primer pairs OI1 and OI2c (Table 1). PCR reactions were conducted under the thermal cycling conditions described in Table 2. The expected amplicon size was approximately 1160 bp.

Gel Electrophoresis

PCR products were analyzed by electrophoresis using 1% agarose gel prepared in 1× TAE buffer. A volume of 5 µL PCR product was loaded into each well along with a DNA ladder as a molecular size marker. Electrophoresis was performed at 100 V for 30 minutes. DNA bands were visualized under UV transillumination to confirm the presence of the expected PCR amplicon.

DNA Sequencing and Molecular Identification

Positive PCR products were subjected to Sanger sequencing. The obtained nucleotide sequences were edited and assembled using BioEdit and CLC Genomics Workbench, and subsequently analyzed using the Basic Local Alignment Search Tool (BLAST) against the NCBI GenBank database to determine sequence similarity. Molecular identification of *Candidatus Liberibacter asiaticus* was based on the highest percentage of sequence similarity with reference sequences. Phylogenetic analysis was conducted using MEGA 11 software based on the Neighbor-Joining method with 1,000 bootstrap replications to evaluate the evolutionary relationships between the obtained sequences and reference strains.

Table 1. Primers used for PCR amplification of *Candidatus Liberibacter*

Primer	Sequence (5'–3')	Target gene	Primer source	Amplicon size (bp)
OI1 (Forward)	CGT ATG CAA TAC GAG CGG C	16S rRNA	Jagoueix *et al.* (1996)	1160
OI2c (Reverse)	GCC TCG CGA CTT CGC AAC CCA T	16S rRNA	Jagoueix *et al.* (1996)	

Table 2. PCR thermal cycling conditions

Step	Temperature (°C)	Time	Cycles
Initial denaturation	92	30 s	1
Denaturation	92	60 s	40
Annealing	60	30 s	
Extension	72	90 s	
Final extension	72	90 s	1

RESULTS AND DISCUSSION

Detection of CVPD by PCR Analysis

A total of four leaf samples of *Citrus reticulata* showing CVPD-like symptoms were collected from different locations, namely Tejakula, Tukad Sumaga, Pecatu, and Balai Benih Induk Luwus. Molecular detection of CVPD was carried out using PCR with primers targeting the 16S rRNA gene of *Candidatus Liberibacter asiaticus*.

Amplification results indicated that only two of the four samples generated DNA bands of the expected size at approximately 1,160 bp, indicating positive detection of CVPD (Figure 2). The positive samples were obtained from Pecatu (Lane 1) and Tukad Sumaga (Lane 2). In contrast, no amplification was observed in Lane 3 and Lane 4, corresponding to samples from Tejakula and Balai Benih Induk Luwus, respectively, and these samples were therefore considered negative.

Sequence and Phylogenetic Tree Analysis of 16S rRNA Gene

PCR-positive samples were subjected to Sanger sequencing for molecular identification. The obtained nucleotide sequences were edited and assembled using BioEdit and CLC Genomics Workbench. Sequence similarity analysis was conducted using the BLASTn program available on the National Center for Biotechnology Information (NCBI) GenBank database.

BLAST analysis revealed that the Pecatu isolate showed 100% sequence identity, while the Tukad Sumaga isolate showed 99.81% sequence identity to *Candidatus Liberibacter asiaticus* reference sequences, with 100% query coverage and an E-value of 0 for both samples (Table 3). These results confirmed that the amplified PCR products corresponded to *Candidatus Liberibacter asiaticus*.

Phylogenetic analysis based on partial 16S rRNA gene sequences further supported the BLAST results. The Neighbor-Joining phylogenetic tree was constructed using partial 16S rRNA gene sequences from this study and reference sequences retrieved from the NCBI GenBank database, showing that both isolates (CP and CTS) clustered closely with Candidatus Liberibacter asiaticus. The Neighbor-Joining phylogenetic tree showed that both isolates (CP and CTS) clustered closely with *Candidatus Liberibacter asiaticus* reference sequences retrieved from the NCBI GenBank database, forming a well-supported clade with high bootstrap values (Figure 3). This clustering indicates a close evolutionary relationship between the isolates obtained in this study and previously reported *Candidatus Liberibacter asiaticus* strains.

Table 3. BLAST analysis of 16S rRNA sequences from CVPD-positive samples

No	Sample Code	Accession (NCBI)	Reference sequence description		Query Cover (%)	Identity (%)	E-value
1	CP	KJ944267.1	*Candidatus asiaticus*	*Liberibacter*	100	100	0
2	CTS	OL589173.1	*Candidatus asiaticus*	*Liberibacter*	100	99.81	0

Note: CP = sample from Pecatu; CTS = sample from Tukad Sumaga.

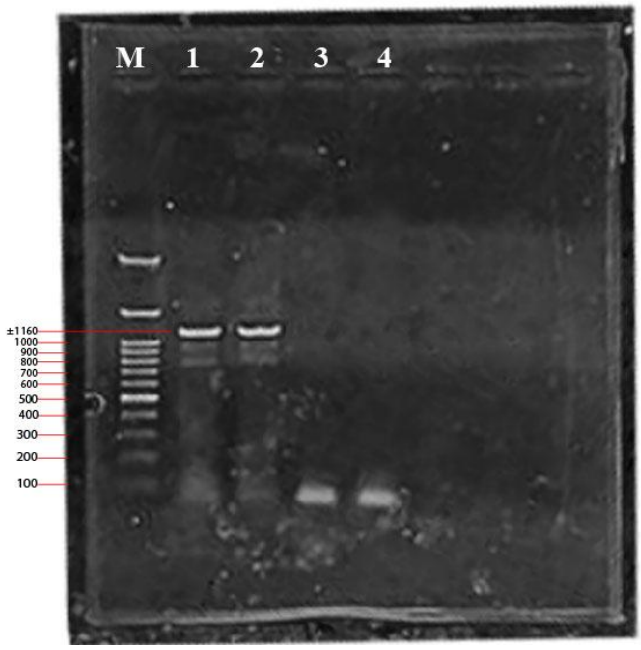


Figure 2. PCR detection of *Candidatus Liberibacter asiaticus* based on 16S rRNA gene amplification from mandarin citrus (*Citrus reticulata* var. Tejakula) leaf samples. Lane M: 1 kb DNA ladder; Lane 1: Pecatu (positive); Lane 2: Tukad Sumaga (positive); Lane 3: Tejakula (negative); Lane 4: Balai Benih Induk Luwus (negative). A DNA band of approximately 1,160 bp indicates a positive result

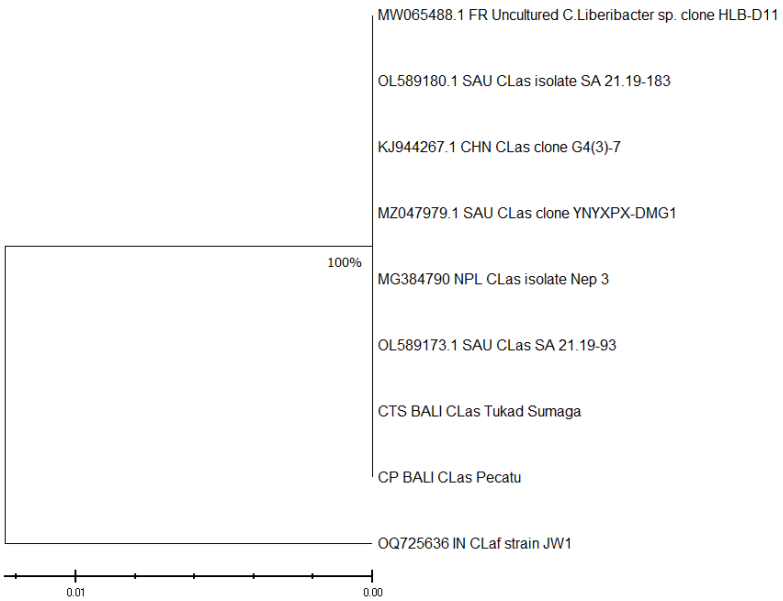


Figure 3. Neighbor-Joining phylogenetic tree based on partial 16S rRNA gene sequences showing the relationship between Bali isolates (CP and CTS) and reference sequences of *Candidatus Liberibacter asiaticus* from GenBank. Bootstrap values (1,000 replicates) are shown at the nodes. The scale bar indicates nucleotide substitutions per site

Discussion

The molecular detection results demonstrated that *Candidatus Liberibacter asiaticus* (CLas) was only identified in two out of the four sampled locations, indicating that the distribution of CVPD in the surveyed areas was not uniform. This finding suggests that the presence of visual symptoms alone is not sufficient to confirm infection, as similar symptoms may arise from other factors such as nutrient imbalance or environmental stress. Therefore, reliance on molecular methods is crucial to ensure accurate diagnosis of CVPD in citrus plants.

Differences in detection results among locations may reflect variations in cultivation practices and local environmental conditions. Orchards with more intensive management, including regular monitoring and better plant health practices, are likely to suppress disease development. In contrast, areas with less intensive management may provide more favorable conditions for pathogen establishment and spread. Although these factors were not quantitatively measured in this study, they could contribute to the observed differences in CLas detection across sampling sites.

The sequencing analysis provided strong confirmation of the PCR results, as the obtained sequences showed very high similarity to reference sequences of CLas available in public databases. This high level of similarity indicates that the detected pathogen corresponds to previously reported CLas strains. Furthermore, phylogenetic analysis revealed that the isolates from Bali were closely grouped with other CLas sequences, forming a well-supported cluster. This pattern suggests a close genetic relationship and supports the notion that CLas populations tend to exhibit limited variation, particularly when analyzed using the 16S rRNA gene.

The relatively conserved nature of the 16S rRNA gene explains the high similarity observed among isolates from different regions. While this marker is highly reliable for confirming pathogen identity, its resolution at finer genetic levels is limited. Nevertheless, for diagnostic purposes, especially in regions with limited access to advanced genomic approaches, 16S rRNA-based detection remains a practical and dependable method.

Overall, the results of this study confirm that CLas is present in mandarin citrus var. Tejakula in Bali and demonstrate that PCR combined with sequencing analysis is an effective approach for its detection. The findings also highlight the importance of integrating molecular techniques into disease monitoring programs to improve the accuracy of CVPD diagnosis. This information is expected to support future efforts in disease management and contribute to the sustainability of citrus production in affected regions.

CONCLUSION

Based on molecular detection and sequence analysis, Candidatus Liberibacter asiaticus (CLas), the associated pathogen of Citrus Vein Phloem Degeneration (CVPD), was confirmed in mandarin citrus var. Tejakula (Citrus reticulata var. Tejakula) from Bali. PCR amplification targeting the 16S rRNA gene successfully detected CLas in two out of four symptomatic samples, originating from Pecatu and Tukad Sumaga, with an expected amplicon size of approximately 1,160 bp. Sequence analysis using BLAST showed high similarity with reference CLas sequences, with 100% identity for the Pecatu isolate and 99.81% identity for the Tukad Sumaga isolate. Phylogenetic analysis further confirmed that both Bali isolates were closely grouped with Candidatus Liberibacter asiaticus reference strains. These findings demonstrate that PCR combined with 16S rRNA gene sequencing is a reliable approach

for accurate detection and molecular identification of CLas in mandarin citrus var. Tejakula. The results highlight the importance of molecular diagnostics as part of CVPD monitoring and management strategies to support sustainable citrus production in Bali.

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