Journal of Biology, Agriculture and Healthcare
ISSN 2224-3208 (Paper) ISSN 2225-093X (Online)
Vol.8, No.10, 2018

New Strains of Candidatus Liberibacter asiaticus in Citrus Plants in Bali

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Abstract
Candidatus Liberibacter asiaticus is the most widely known bacteria causing citrus greening (Huanglongbing) from three species of *Candidatus Liberibacter*. This study was conducted to ascertain the phylogenetic relationship between *Can. Liberibacter asiaticus* Indian isolates which have a higher diversity in 16S rDNA with Asian isolates in general. An citrus leaves samples with CVPD disease symptoms were collected from several locations in Bali. Detection of *Can. L. asiaticus* in the sample was performed by PCR using a 16S rDNA-specific primer F: 0I1 and R: 0I2c. Based on the DNA amplification results, six samples taken from *Citrus nobilis* reacted positively, and each one taken from *C. amblycarpa*, *C. aurantium* and *C. grandis* were also reacted positively. DNA of nine positive samples were purified and sequenced. The sequencing results showed a close relationship between *C. nobilis* samples with *C. aurantium* at a distance of 0.000, but the relationship is relatively far away with the other seven samples and with strains of Poona India, *L. africanum* strains of Nelspruit South Africa and nine isolates *Can. L. asiaticus* accessed from GenBank NCBI is with a distance of 0.600-0.603. Two samples (JBC1311 and JBC1351) are different from Indian Poona strain in Purine base positions with Pyrimidine, and Pyrimidine base positions with Purines in the 16S rDNA sequence. Based on these results, one sample each taken from *Citrus nobilis* JBC1311) and *C. aurantium* (JBC1351) are considered as new strains and different from other strains occurred in Bali, and also different from the strains of Poona (India) and the strain of Nelpruit (South Africa), while 7 (seven) other samples are the same strains.
Keywords: Citrus greening ,CVPD, *Candidatus Liberibacter asiaticus*, 16S rDNA

1. Introduction

Citrus greening disease (huanglongbing) in Indonesia is named citrus vein phloem degradation which is shortened to CVPD (Wirawan *et al*., 2004). The disease was first reported in Indonesia in the early 1960s and became the most serious disease threatening citrus production in Indonesia (Nurhadi, 2015). In South China, CVPD disease was reported 90 years ago (Reinking, 1919). The disease-causing pathogen is a gram-negative bacteria, limited only to phloem, which is a new genus, *Candidatus Liberibacter* placed on the Proteobacteria subdivision (based on 16S rDNA sequence) (Jagoueix *et al*., 1994). Infection of the plant occurs through two Psyllid vector insects i.e, *Diaphorina citri* in Asia and *Trioza erytreae* in Africa (Jagoueix *et al*., 1997). According to Jagoueix *et al*. (1994) sequence analysis of 16S rDNA in *Can.L. asiaticus* is used for the diagnosis of the disease and to estimate the genetic diversity of *Can.L. asiaticus* that spread all over the world. Based on a 16S rDNA analysis most bacterial isolates were *Can.L. asiaticus* which are similar or identical (Subandiyah *et al*., 2000; Wirawan *et al*., 2003).

Phylogenic analysis of 16S rDNA sequences and single nucleotide polymorphisms in the *omp* gene sequence revealed that North Indian isolates were genetically closer to Asian isolates such as Japan, Taiwan and Vietnam. Strain *Can.L. asiaticus* Asia is commonly present in Northeast India (Miyata *et al*., 2011). Based on an amplified DNA sequence analysis with a specific primer of 16S rDNA (0I1 and 0I2c) samples with chlorosis symptom taken from Bali, Bima, Cianjur and Tulungagung reacted positively. A comparison of 16S rDNA sequences from the area with *L. asiaticus* strains of Indian Poona and *L. africanum* strains of Nelspruit South Africa revealed a genetic variation in the form of basic differences, base insertions, basic deletions and the possibility of base inversion, and no genetic linkage to symptom patterns of leaf chlorosis (Zubaidah, 2004). *L. asiaticum* bacteria that causes CVPD disease in Indonesia consists of several different strains. This difference is indicated by the sequence difference of the nucleotides from the amplified 16S rDNA fragment. The bacterial strains that cause CVPD disease in citrus plants in West Kalimantan, Cianjur, Tulungagung, Bali, Bima and South Sulawesi showed differences (Wirawan, *et al*., 2003).

The objective of this study is to know the closeness of sequence relationship of 16S rDNA samples of *Can.L. asiaticus* obtained from citrus plants in Bali, and relationship with strains of Poona India and Nelspruit South Africa.

2. Research Methods

2.1. Sample Collection

A total of 15 leaves samples were collected from seven locations in Bali. Samples were taken from five types of citrus namely *Citrus nobilis* cv.*microcrva* (Siam), *C. nobilis* cv.*chrysocarva* (Keprok Selayar, Keprok Besakih,

Keprok Batu55), *Citrus Amblycarpa* (Limo), *Citrus aurantium* (Seponten) and *Citrus grandis*. *Citrus nobilis* cv.*microcrva* were grown in all sampling locations while others scattered in certain location. Based on visual observation, there was a variation of CVPD disease symptoms in particular the pattern of chlorosis among types of citrus and sampling locations.

Variation in CVPD symptom was determined based on the content of chlorophyll. Five citrus leaves were taken for total DNA isolation. The leaves were placed on a table and sequenced from number 1 to 5. The chlorophyll content of each leaf was measured using chlorophyll meter (SPAD 502 serial No. device. 7681-3011) as much as 10 times so as to obtain an average per leaf. The leaf blade of each leaf was then used for DNA extraction and isolation.

2.2. DNA Extraction

DNA from all citrus leaves samples were extracted using 'Genomic DNA Kit from Plant Maccherey-Nagel' conducted at the Genetic and Biomolecular Resource Laboratory, Udayana University, Denpasar. A total of 100 mg of the mother's bone leaves were cut into small pieces and added with liquid Nitrogen and crushed until smooth in a mortar. The sample was put into an Eppendorf tube, added with 400 µl GP1 and 5 µl RNase, lysis buffer, and then was mixed using vortex. Sample was incubated at 60°C for 10 minutes. During the incubation, the tube was inverted every 5 minutes. At the same time, a 200 µl per sample of elusion buffer was heated at 60 ^{o}C. This elusion buffer was used for 4 stages of DNA elusion. Sample was added with 100 µl GP2 buffer then was mixed on vortex and incubated on ice for 3 minutes, then was transferred into filter column which has been installed on 2 ml of collection tube, then was centrifuged at 1000 x g for 1 minute. The filter is removed. Supernatant was transferred into a new Eppendorf tube and added with 1.5 times GP3 buffer and mixed on a vortex until homogeneous. A visible pellet was then suspended using a pipette. A total of 700 µl of the mixture was transferred into a GD column that had been installed in the collection tube, and then was centrifuged at 14,000 -16,000 x g for 2 minutes. The supernatant was discarded. GD columns that have been placed on 2 ml collecting tubes was added with 400 µl W1 buffer into GD columns then centrifuged at 14,000 -16,000 x g for 30 seconds. The liquid was removed and the GD column was reassembled in 2 ml of the collection tube, then a 600 µl washed buffer was added to GD columns and centrifuged at 14,000 -16,000 x g for 30 seconds. The liquid is removed and the GD column is reassembled in 2 ml of collection tube and then centrifuged 14,000 -16,000 x g for 30 seconds for drying process. Move the dried GD columns to the Eppenorf tube, then added with 100 µl elusion buffer right in the middle of GD columns and stand for 3-5 minutes, then centrifuged at 14,000 - 16,000 xg for 30 seconds , then the DNA was obtained for further test.

2.3. DNA amplification

DNA was amplified using specific 16S rDNA primers. Forward Primer was OI1: 5'GCG CGT ATG CAA TAC GAG CGG C 3 'and reverse primer was OI2c: 5' GCC TCG CGA CTT CGC AAC CCA T 3 '. PCR program used was: Pre-treatment at temperature 92 ^{o}C for 30 seconds for one cycle. Denaturation at 92 °C for 60 seconds; annealing (tempering primer on template DNA) at 60 °C for 30 seconds and Elongation (DNA synthesis) at 72 °C for 90 seconds for 40 cycles. Extention (adjustment of DNA double threads) at 72 °C for 90 seconds for one cycle. Visualization of PCR-amplified DNA was done as follows. PCR-treated DNA fragments of 1 µl were electrophoresed on 1% TBE agarose gel. Buffer for electrophoresis used was 1% TAE buffer containing 40 mM sodium EDTA. Electrophoresis was carried out at 100 volts for 1-2 hours (Sambrook *et al*, 1989).

2.4. Sekuensing 16S rDNA

Amplified DNA was then purified and subjected to sequencing process. The sequencing was performed using Automatic Flourescent DNA Sequencer (ABI 377A) and was done at Genetics Science Indonesia Ltd. West Jakarta, Indonesia. The sequencing results were then compared with the existing data on GenBank using BLAST-N (Basic Local Alignment Search Tool-Nucleotide) accessed from the NCBI (National Center for Biotechnology Information) program. The arrangement of phylogeny trees was done using MEGA 5.10.

3. Results and Discussion

The results of total isolation of citrus tree DNA from 15 samples showing symptoms of chlorosis, indicated positive result for DNA content. This data suggested that total DNA of plants has been well isolated. Total isolation of citrus tree DNA needs to be done because the bacteria CVPD can not be cultured and to obtain a good quality of DNA template. According to Taylor (1993), in amplification with PCR required good quality of DNA template. Ohtsu *et. al*. (2002) stated that because the bacteria of CVPD is still can not be cultured so it is not possible to isolate bacterial DNA directly, then the approach is to isolate the plant DNA.

The amplification results using the specific primes of 16S rDNA (F: 0I1 and R: 0I2c) obtained four samples reacting positively in *C. nobilis* cv. *microcrva*, two samples reacted positively in C. *nobilis* cv. *chrysocarva*, and in the *C. amblycarpa*, *C. aurantium* and *C. grandis* samples reacted positively as well. The CPVD symptom was

found in all sampling sites, but not all types of citrus were infected with CVPD, as in *C. nobilis* cv. *microcrva* from Abuan, Mangguh and Bayung villages did not react positively, likewise, at the *C. nobilis* cv. *chrysocarva* i.e. two samples from Katung village and one sample from Bayung village (Table 1 and Fig. 1). According to Adiartayasa (2006) PCR analysis results using specific primes 16S rDNA on citrus leaf *C. nobilis* cv. *chrysocarva* (Keprok Batu-55 and Keprok Cina) Kintamani and Keprok Selayar (Sekar Mukti) and *C. nobilis* cv. *microcarva* (Siam Kintamani) is positively infected with CVPD disease, while in *C. nobilis* cv.*chrysocarva* (Keprok Besakih, Mulung and Selayar) Kintamani is negative for CVPD disease infection. Zubaidah (2004) reported no consistency between the symptoms of chlorosis and the presence of CVPD bacteria. The adjacent leaves and consecutive leaves in a single branch do not always contain bacteria, suggested that bacteria is not distributed systemically in the plant.

Himawan *et al*. (2010) reported huanglongbing symptoms on *C. nobilis* cv. *microcrva* leaves are categorized into 8 types ranging from unseen symptoms, some types of chlorosis to the symptoms of thickening of the leaf bones. All types of single samples of mother bone leaves tested contained *Can. L. asiaticus* when detected using PCR technique with specific primers MHO 353 and MHO 354. Wijaya (2003) reported that infection in citrus and yellow plants can be detected by PCR method. Adults and nymphs of *Diaporina citri* living on citrus plants infected with positive CVPD bacteria contain *L. asiaticus*. Sritamin (2007) confirmed CVPD infection in Siam Bangli citrus leaves and bacterial vector, *D. citri* indicated by the presence of DNA band with 1160 bp in size.

The homology level of nine citrus samples reacting positively is 99% with *L. asiaticus* strain Poona India and 97% with *L. africanum* strain Nelspruit South Africa (Table 2). The relationship of the two samples (JBC1311 and JBC1351) has a range of 0,000 and their relationship with seven samples (JBC1313, JBC1315, JBC1317, JBC1323, JBC1341, JBC1324, and JBC1361) has a distance of 0.602; with Indian Poona strain has a distance of 0.600; with *L. africanum* strain Nelspruit South Africa has a distance of 0.601 and nine *Can.L. asiaticus* isolates in GenBank NCBI has a distance of 0.602-0.603 (Figure 2). So the smaller the closer the relationship among bacteria, while the greater the distance value, the farer the relationship among bacteria. According to Hagstrom *et al*. (2002), when the homologous level of the 16S rDNA sequence is equal to or greater than 97% expressed as the same type, thus the bacteria of seven isolates (JBC1313, JBC1315, JBC1317, JBC1323, JBC1341, JBC1324, and JBC1361 are *Can. L. asiaticus*. While two isolates samples (JBC1311 and JBC1351 are different from seven isolates of Bali Province, strain Poona India, nine Isolates at the NCBI Bank Gene and *L. africanus* strain Nelspruit, South Africa. Wang *et al*. (2012) reported that there is a significant strains variation of *Can. L. asiaticus* from inter and intra-regional diversity.

Table 1. Presences of *Can. L. asiaticus* based on PCR techniques in samples taken from various locations in Bali

No	Sample's code	Sampling sites			Host	Presences of *Can. L. asiaticus**
		Regency	District	Area		
1.	JBC1311	Bangli	Kintamani	Katung	*C. nobilis* cv. *microcarva*	+
2.	JBC1321	Bangli	Kintamani	Katung	*C. nobilis* cv.*chrysocarva*	-
3.	JBC1322	Bangli	Kintamani	Katung	*C. nobilis*cv. *chrysocarva*	-
4.	JBC1312	Bangli	Kintamani	Mangguh	*C. nobilis* cv. *microcarva*	-
5.	JBC1323	Bangli	Kintamani	Mangguh	*C. nobilis cv.chrysocarva*	+
6.	JBC1341	Bangli	Kintamani	Mangguh	*Citrus amblycarpa*	+
7.	JBC1324	Bangli	Kintamani	Mangguh	*C. nobilis cv.chrysocarva*	+
8.	JBC1351	Bangli	Kintamani	Mangguh	*Citrus aurantium*	+
9.	JBC1361	Bangli	Kintamani	Mangguh	*Citrus grandis*	+
10.	JBC1313	Bangli	Kintamani	Blancan	*C. nobilis cv. microcarva*	+
11.	JBC1314	Bangli	Kintamani	Abuan	*C. nobilis cv. microcarva*	-
12.	JBC1325	Bangli	Kintamani	Bayung	*C. nobilis cv.chrysocarva*	-
13.	JBC1315	Gianyar	Tegallalang	Taro	*C. nobilis cv. microcarva*	+
14.	JBC1316	Bangli	Kintamani	Bayung	*C. nobilis cv. microcarva*	-
15.	JBC1317	Gianyar	Tegallalang	Kerta	*C. nobilis cv. microcarva*	+

* + = present; - = not present

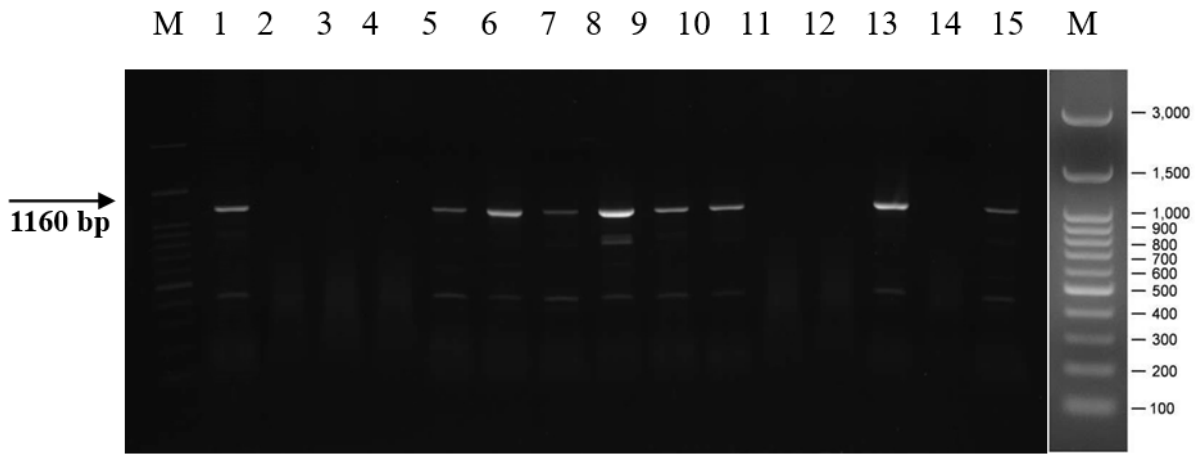

Fig. 1.
PCR amplification of 16S rRNA genes of 15 isolates. M = DNA marker ladder 100 bp.
Arrow indicates PCR products of isolates JBC1311 (column 1), JBC1321 (column 2),JBC1222 (column 3),JBC1312 (column 4), JBC1323 (column 5), JBC1341 (column 6), JBC1324 (column 7), JBC1351 (column 8),JBC1361 (column 9),JBC1313 (column 10), JBC1314 (column 11),JBC1325 (column 12), JBC1315 (column 13), JBC1316 (column 14), and JBC1317 (column 15).

Based on the results of the dendrogram obtained three clusters, namely the first cluster consisted of seven isolates of Bali (JBC1313, JBC1315, JBC1317, JBC1323, JBC1341, JBC1324, and JBC1361), *L. asiaticus* strain Poona India, and nine isolates of *Can. L. asiaticus* on NCBI GenBank, while the second cluster consisted of two isolates of Bali (JBC1311 and JBC1351) and the third cluster was *L. africanus* strain Nelspruit South Africa. These results indicated that there are a variation of pathogenic strains of *Liberobacter* and cause variation of symptoms. In our present study we found two strains of *Can. L. asiaticus* which are different from the strains of Indian Poona and Nelspruit of South Africa (Figure 2).

Table 2. Maximum scores, E values, and percentage of similarities of nine isolates of *Can. L. asiaticus* with other identified strains

Sample's code	Species homolog	Identity	Max Score	Query Cover	E Value	Accession Number
JBC1311	*L. a*. Strain Poona	99%	1024	99%	0.0	L22532.1
	L. af.. Strain Nelspruit	97%	974	97%	0.0	L22533.1
	C. L.a. Strain Mmnd-1	100%	1907	100%	0.0	KC800962.1
	C. L.a. Strain Tnal-9	100%	1907	100%	0.0	KC800958.1
JBC1323	*L. a*. Strain Poona	99%	1015	99%	0.0	L22532.1
	L. af.. Strain Nelspruit	97%	965	97%	0.0	L22533.1
	C. L.a. isolat MHN-127	100%	1891	100%	0.0	KT164831.1
JBC1341	*L. a*. Strain Poona	99%	1016	99%	0.0	L22532.1
	L. af.. Strain Nelspruit	97%	966	97%	0.0	L22533.1
	C. L.a. isolat MHN-127	99%	1891	100%	0.0	KT164831.1
	C. L.a. Strain Mmnd-1	99%	1907	100%	0.0	KC800962.1
JBC1324	*L. a*. Strain Poona	99%	1008	99%	0.0	L22532.1
	L. af.. Strain Nelspruit	97%	956	97%	0.0	L22533.1
	C. L.a. isolat MHN-127	100%	1891	100%	0.0	KT164831.1
JBC1351	*L. a*. Strain Poona	99%	1000	99%	0.0	L22532.1
	L. af.. *Strain Nelspruit*	97%	948	97%	0.0	L22533.1
	C. L.a. Strain Mmnd-1	100%	1907	100%	0.0	KC800962.1
JBC1361	*L. a*. Strain Poona	99%	1006	99%	0.0	L22532.1
	L. af.. Strain Nelspruit	97%	954	97%	0.0	L22533.1
	C. L.a. isolat MHN-127	100%	1891	100%	0.0	KT164831.1
JBC1313	*L. a*. Strain Poona	99%	1012	99%	0.0	L22532.1
	L. af.. Strain Nelspruit	97%	962	97%	0.0	L22533.1
	C. L.a. isolat MHN-127	99%	1891	100%	0.0	KT164831.1
JBC1315	*L. a*. Strain Poona	99%	1022	100%	0.0	L22532.1
	L. af.. Strain Nelspruit	97%	973	97%	0.0	L22533.1
	C. L.a. Strain Tnal-9	99%	1902	100%	0.0	KC800958.1
	C. L.a. Strain Tnal-8	99%	1902	100%	0.0	KC800958.1

Sample's code	Species homolog	Identity	Max Score	Query Cover	E Value	Accession Number
JBC1317	*C. L.a*. Strain Mmnd-1	99%	1907	100%	0.0	KC800962.1
	L. a. Strain Poona	99%	1006	99%	0.0	L22532.1
	L. af.. Strain Nelspruit	97%	954	97%	0.0	L22533.1
	C. L.a. isolat MHN-127	100%	1891	100%	0.0	KT164831.1

Two samples (JBC1311 and JBC1351) are different from Indian Poona strain in Purine base positions with Pyrimidine, and Pyrimidine base positions with Purines in the 16S rDNA sequence (Table 3). In the position of Guanine to the Adenine bases are different in 76 bases, while in the position of Adenine to Guanine are different in 53 bases. The differences are also occurred in other positions as indicated in Table 3. Katoh *et al*. (2014) found several strains of *Can. L. asiaticus* from Japan that lacks of bacteriophage-type region of DNA polymerase (pol DNA), in contrast to the Florida psy62 strain. All of the polymerase genome sequences are negative on *Can. L. asiaticus* Ishi-1 a Japanese isolate that has been determined by metagenomic analysis of DNA extracted from *Can. L. asiaticus* that infects psyllids and leaf bone.

Kuykendall *et al*. (2012) reported that *Can. L. asiaticus* and intracellular obligate bacteria associated with citrus-greening disease also called huanglongbing are members of Rhizobiales along with the micro-nitrogen micro-symbionts such as *Sinorhizobium meliloti*, *Bradyrhizobium japonicum*, *Agrobcterium tumefaciens* and *Bartonella henselae*. The micro-symbiont gene group represents Rhizobiales, having the same gene before being ordered by 16S RNA and represented by three families of *Bartonellaceae*, *Bradyrhizobiaceae* and *Rhizobiaceae*. The circular chromosomes of each of the five species of Rhizobiales examined have less orthologic genes in four other species. For example 63 proteins encoded by *Can. L. asiaticus*is not shared with other members of Rhizobiales. Of these 63 proteins, 17 have predicted functions associated with DNA replication or transcription of RNA, and some may have roles associated with low GC genomic content. Hartung *et al*. (2011) reported that genome of *Can. L. asiaticus* and *Bartonella henselae* drastically reduced the gene content, size and with relatively low levels of Guanine and Cytosine.

Table 3. Position of the differences of the number of Purin and Pyrimidin bases in Strain Poona with other Purin and Pirimidin than Nine the Samples Citrus Bali Provinsi

Isolate code	G-A	A-G	G-C	G-T	A-C	A-T	C-T	T-C	T-A	T-G	C-A	C-G
JBC1323			1									1
JBC1341			1									1
JBC1324			1									1
JBC1361			1									1
JBC1313			1									1
JBC1315			1									1
JBC1317			1									1
JBC1311	76	53	68	67	66	36	56	77	26	31	34	19
JBC1351	76	53	68	67	66	36	56	77	26	31	34	19

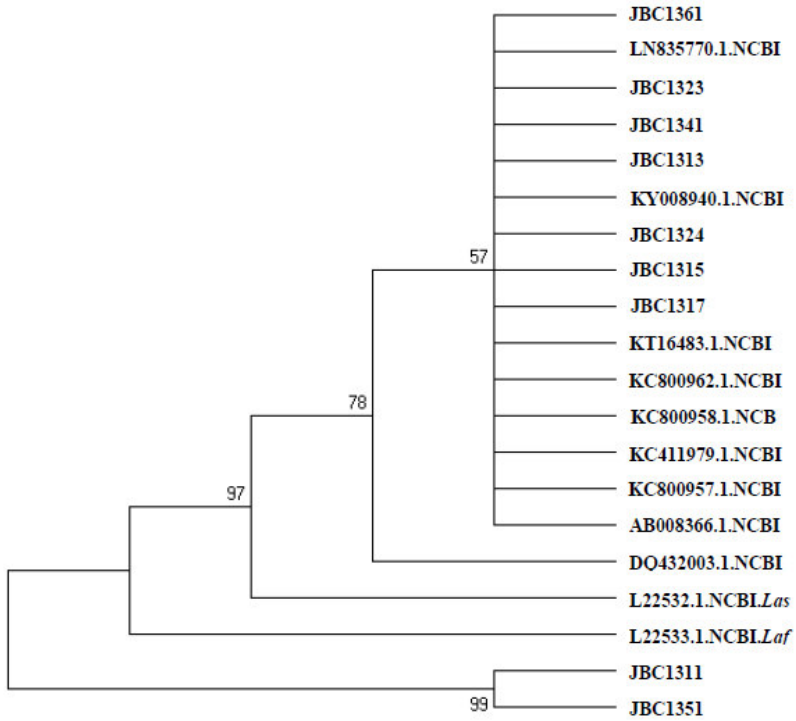

Fig. 2.
The Phyllogenetic relationship between nine isolates of *Can. L. asiaticus* i.e JBC1311, JBC1313, JBC1315, JBC1317, JBC1323, JBC1324, JBC1341, JBC1351, JBC1361, with strain Poona India, *L. africanus* strain Nelspruit, South Africa, and nine isolates of *Can. L. asiaticus* on Data GenBank NCBI

4. Conclusion

From 15 samples of citrus leaves from Bali Province that showed CVPD disease symptom, nine samples reacted positively on Specific Primer 16S rDNA (f: OI1 and r: OI2c). All areas of the citrus plants indicate the presence of CVPD infection, but not all citrus species are infected with CVPD disease. The phylogenetic relationship between bacteria showed close association between JBC1311 and JBC1351 samples, but the relation was relatively far away with seven samples (JBC1313, JBC1315, JBC1317, JBC1323, JBC1324, JBC1341 and JBC1361), *L. asiaticus* strain Poona India, *L. africanum* strain Nelspruit South Africa and Nine Isolat *Can. L. asiaticus*on GenBank NCBI. The JBC1311 and JBC1351 samples are considered as new strains of *Can. L. asiaticus* in citrus in Bali**.** They are also distinct from the strains of Poona (India) and the Nelpruit strain (South Africa).

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