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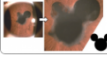
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Research Article

Gene polymorphisms CVPD^r on some plants citrus in Bali Island

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Abstract

Citrus Vein Phloem Degeneration (CVPD) is the main disease of citrus plants in Indonesia. This disease is caused by Gram negative bacteria, *Candidatus Liberibacter asiaticus*. Almost all citrus plants are susceptible to this disease and only a few citrus plants such as seedless lime (*Citrus aurantiifolia* var. Seedless) and kinkit citrus (*Triphacia trifoliata*) are tolerant. Both of these citrus plants store DNA fragments of CVPD^r which are considered as tolerant factors (841 bp). However, this study found that CVPD^r DNA fragments were also found in citrus plants susceptible to CVPD disease. This research aims to study DNA polymorphisms from CVPD^r DNA fragments in citrus plants on the island of Bali. The PCR test showed *T. trifoliata* and *C. aurantiifolia* that are resistant to CVPD and Phylogenically are in the same group as *C. nobilis* var Buleleng, *C. reticulata* var. Slayer Buleleng, and *C. amblycarpa*. On the other hand, citrus plants susceptible to CVPD are in a different group. There are two types of citrus plants not containing CVPD^r DNA fragments, namely *C. nobilis* var. Petang and *M. paniculata* L. These results indicate that the CVPD^r DNA fragment polymorphism is a factor tolerant to CVPD disease.

Introduction

Citrus is one of the horticultural plants that is very important in the economy of the people of Indonesia. This plant has long been known and cultivated in Indonesia [1]. Citrus is a local fruit that is pretty much on the market, the quality of citrus fruit can be influenced by various factors including the attack of *Citrus Vein Phloem Degeneration* (CVPD). All cultivated citrus plants are susceptible to CVPD disease attacks [2]. On the other hand it was reported that several types of citrus plants, especially those with less economic value, were known to be resistant to CVPD. Types of citrus that are resistant to CVPD, hereinafter referred to as citrus with the CVPD^r gene. Citrus plants resistant to CVPD (CVPD^r) are thought to contain genes that produce a trait that is able to break the pathogenic infection of CVPD (*L. asiaticum*) or is able to resist transmission of pathogens carried by vector insects [3].

One gene named CVPD^r gene plays a role in citrus resistance of CVPD. The CVPD^r gene is found in citrus plants that are resistant or relatively resistant to CVPD diseases, namely Kinkit citrus and Seedless Lime citrus. But CVPD^r gene is also found in citrus Siam Kintamani, which is a citrus crop that is susceptible to disease CVPD, so it becomes difficult to explain the mechanism of disease infection CVPD. It is possible that the CVPD^r gene is not expressed so it cannot play a good role in providing resistance to CVPD disease. The CVPD^r gene functions to remove obstacles in the absorption of nutrients

into plant cells by the CVPD bacteria. The CVPD^r gene is located on the chromosome of an citrus plant, but its molecular-level mechanism of action is unknown [3].

This study aims to determine the pathogenic CVPD in various types of citrus plants in Bali Indonesia. This study uses analysis *Polymerase Chain Reaction* (PCR) with O₁₁ and O₁₂ primers and amplified DNA fragments will be sequenced. The sequence of DNA fragments from various types of citrus plants in Bali will be analyzed to see polymorphisms that may be related to CVPD infection rates. Among all the cultivation of citrus such as Tangerines citrus, Siam citrus, Lime citrus, Lime Without Seeds citrus and other citrus fruits, are searched for the presence of genes CVPD^r using PCR with primers and gene CVPD^r [4].

Material and methods

Type of research and sample sources

This research is an experimental study conducted at the Biotechnology Laboratory, University of Udayana, Bali-Indonesia. Determination of the sample in this study using samples of citrus leaves that are resistant and susceptible to CVPD disease from several types of citrus plants namely kinkit (*Triphacia trifoliata*), seedless lime (*C. aurantiifolia* var. Seedless), siam citrus (*C. nobilis*), tangerines (*C. reticulata*) and limes (*C. amblycarpa*) taken at the center of citrus plantations in Bali.

More Information

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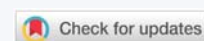
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Keywords: Citrus; CVPD^r gene; Polymorphism



DNA isolation and DNA analysis by PCR

Total DNA from citrus plants was isolated using the NucleoSpin® Plant II kit from Marchery-Nagel. Samples were taken from leaf bones by observing the symptoms of CVPD attacks and detection using PCR analysis. Detection of the presence of the gene CVPD^r performed by PCR analysis using WR-F and WR-R and use the program denaturation at a temperature of 94 °C for 60 seconds, annealing at a temperature of 60 °C for 30 seconds, and elongation at a temperature of 72 °C for 90 seconds. All of these stages were carried out with 36 repetition cycles [3].

Agarose gel electrophoresis and PCR visualization

Agarose gel consists of 1% agarose dissolved in 100 ml TAE buffer (consisting of 40 mM tris acetate pH 7.9; 2 mM Sodium EDTA). DNA samples (8 µl DNA + 2 µl loading dye) each were filled in the gel pit. Electrophoresis is performed with a voltage of 100 volts for ± 20 minutes. Soak in EtBr solution for ± 15 minutes. Then the results of the electrophoresis were visualized with a UV transilluminator to see the position of the bands DNA of each sample and then documented.

CVPD^r gene expression by DNA sequencing analysis

Sequencing analysis Modern automated DNA sequencing machines are able to sort 384 fluorescent-labelled samples at once in a single batch (electrophoresis) that can be carried out up to 24 times a day. This includes only the process of separation and the process of reading the curve, sequencing reaction, cleaning and dissolving in a suitable buffer solution must be done separately. To obtain labelled reaction results that can be detected from printed DNA, the method of "cycle sequencing" is most commonly used. In this method are carried out successively primary annealing, extension by DNA polymerase and denaturation (melting) strands of printed DNA repeatedly (25-40 rounds). The main advantage of cycle sequencing is that it is more efficient. Each step in cycle sequencing is carried out by changing the reaction temperature using a thermal cycler PCR [5].

Results and discussion

Topographically, the average sampling location is at an altitude of 800 meters above sea level. Judging from the climate, there is still a tropical climate which generally consists of five months of the rainy season and seven months of the dry season, and the air condition is quite fresh and clean because the pollution factor is still relatively low. The average annual rainfall is between 2000 and 2500 mm, while the average minimum air temperature is 24 °C and a maximum of 32 °C with climate, rainfall and air temperature factors indicating moderate conditions so that the flora and fauna life is very good.

Detection of CVPD^r fragment distribution by PCR on several types of citrus plants showed that there were differences between susceptible and resistant citrus plants. Vulnerable citrus plants showed positive results containing CVPD, but

also showed positive results containing CVPD^r, as explained in table 1.

PCR analysis results showed that from 27 samples of citrus plants, as many as 25 samples contained DNA fragments of CVPD^r (841 bp). However, only 2 citrus species are resistant to CVPD disease, namely *T. trifoliata* and *C. aurantifolia* var. *Seedless* citrus. On the other hand there are citrus plants that are prone to CVPD. The study also found that two samples from 27 citrus plants did not contain DNA fragments of CVPD^r, namely *C. nobilis* var. *Petang* and *M. paniculata* L. PCR analysis results are presented in figures 1,2.

Research polymorphisms of genes CVPD^r on some citrus plants using a sample of citrus plants in Bali, as a control used citrus *T. trifoliata*, and lemon without seeds (*C. aurantifolia* var. *Seedless*) which is known as a citrus plant which is resistant to CVPD attacks. To get the CVPD^r gene polymorphism among several citrus plants in Bali sequencing was done on the PCR results using primers *forward* (1F): GACTAGGTGGTAATAACTACTTTT and primers *reverse* (1R): CCTTTTGGTCTATCTTTACTTAG and bas is a phylogenetic tree reconstruction method that uses e amplified from the PCR process were 841 bp. Total DNA isolation using the Mini Kit Plant from the nucleoSpin® Plant II from Marchery-Nagel. In the PCR reaction, was used 1 ng DNA samples, primers of 100 mole P011 and P012c, 2µl dNTP, 2µl PCR Buffer (10X), 0.2 Taq polymerase (5 U/µl). The PCR program uses Pre-treatment at

Table 1: Results of Analysis of Several Citrus Plants on the distribution of CVPD and CVPD^r fragments.

No.	Type of Citrus	CVPD	CVPD ^r
1	<i>C. nobilis</i> var. Kintamani	+	+
2	<i>C. grandis</i> L. Besakih	+	+
3	<i>C. grandis</i> L. Kintamani	+	+
4	<i>C. grandis</i> L. Bedugul	+	+
5	<i>C. nobilis</i> var. Bedugul	+	+
6	<i>C. reticulata</i> var. Slayer Mangguh	+	+
7	<i>C. reticulata</i> var. Slayer Kintamani	+	+
8	<i>C. reticulata</i> var. Gianyar tangerine	+	+
9	<i>C. reticulata</i> var. Keprok Petang	+	+
10	<i>C. nobilis</i> var. Gianyar	+	+
11	<i>C. reticulata</i> var. Tangerines Karangasem	+	+
12	<i>C. reticulata</i> var. Keprok Bangli	+	+
13	<i>C. nobilis</i> var. Mangguh	+	+
14	<i>C. nobilis</i> var. Denpasar	+	+
15	<i>C. reticulata</i> var. Keprok Mangguh	+	+
16	<i>C. reticulata</i> var. Tangerines Besakih	+	+
17	<i>C. nobilis</i> var. Tabanan	+	+
18	<i>C. nobilis</i> var. Pecatu	+	+
19	<i>C. nobilis</i> var. Payangan	+	+
20	<i>Triphasia trifoliata</i>	-	+
21	<i>C. reticulata</i> var. Slayer Buleleng	+	+
22	<i>C. amblycarpa</i>	+	+
23	<i>C. aurantifolia</i> var. Seedles	-	+
24	<i>C. nobilis</i> var. Buleleng	+	+
25	<i>C. aurantifolia</i>	+	+
26	<i>C. nobilis</i> var. Petang	+	-
27	<i>Murraya paniculata</i> L.	+	-

Note: + (containing); and - (not containing).

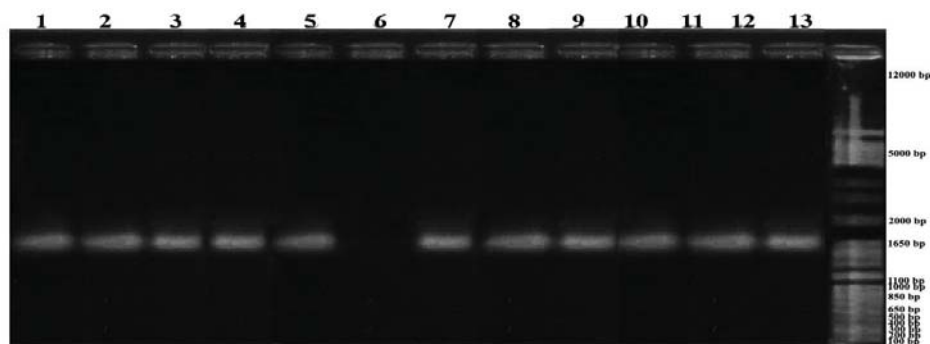


Figure 1: Citrus PCR Results: 1. *C. grandis* L. var. Kintamani, 2. *C. nobilis* var. Bedugul, 3. *C. grandis* L. var. Bedugul, 4. *C. reticulata* var. Keprok Petang, 5. *C. grandis* L. var. Besakih, 6. *C. aurantifolia* var. Seedles (not detected), 7. *C. aurantifolia*, 8. *C. amblycarpa*, 9. *C. reticulata* var Keprok Bangli, 10. *C. reticulata* var. Slayer Mangguh, 11. *C. nobilis* var. Mangguh, 12. *C. reticulata* var. Keprok Mangguh, 13. *C. reticulata* var. Keprok Besakih.

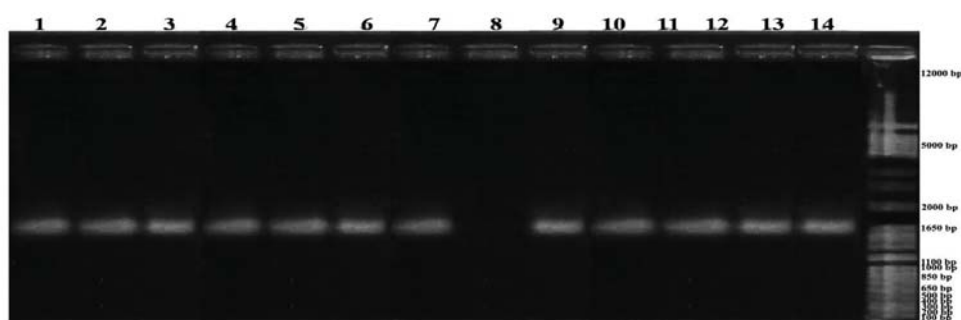


Figure 2: Results of PCR citrus plant samples: 1. *C. nobilis* var. Petang, 2. *Muraya paniculate* L., 3. *C. reticulata* var. Keprok Karangasem, 4. *C. nobilis* var. Kintamani, 5. *C. nobilis* var. Denpasar, 6. *C. nobilis* var. Buleleng, 7. *C. nobilis* var. Siam Gianyar, 8. *T. trifoliata*, (not detected), 9. *C. nobilis* var. Pecatu, 10. *C. reticulata* var. Buleleng Slayer, 11. *C. nobilis* var. Payangan, 12. *C. reticulata* var. Slayer Kintamani, 13. *C. nobilis* var. Tabanan, 14. *C. reticulata* var. Keprok Gianyar.

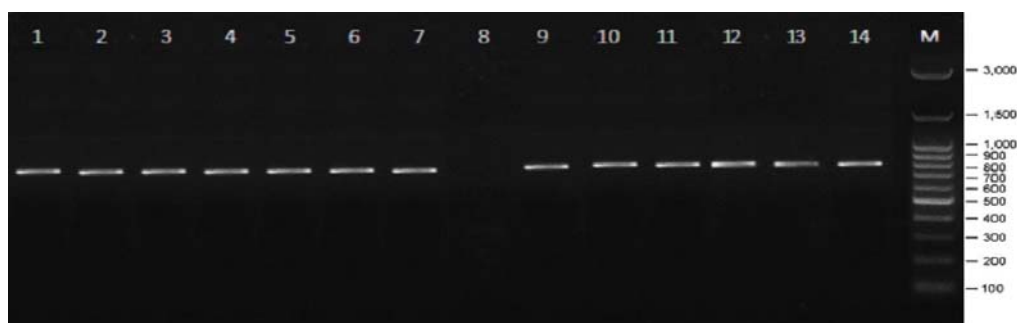


Figure 3: PCR results of citrus plant samples: 1. *T. trifoliata*, 2. *C. aurantifolia* var. Seedles, 3. *C. reticulata* var. Keprok Karangasem, 4. *C. nobilis* var Kintamani, 5. *C. nobilis* var Denpasar, 6. *C. nobilis* var Buleleng, 7. *C. nobilis* var Siam Gianyar, 8. *C. nobilis* var Petang (not detected), 9. *C. nobilis* var Pecatu, 10. *C. reticulata* var Buleleng Slayer, 11. *C. nobilis* var Payangan, 12. *C. reticulata* var Slayer Kintamani, 13. *C. nobilis* var Tabanan, 14. *C. reticulata* var Keprok Gianyar.



Figure 4: Citrus PCR Results: 1. *C. grandis* L. var. Kintamani, 2. *C. nobilis* var. Bedugul, 3. *C. grandis* L. var. Bedugul, 4. *C. reticulata* var. Keprok Petang, 5. *C. grandis* L. var. Besakih, 6. *M. paniculata* L. (not detected), 7. *C. aurantifolia*, 8. *C. amblycarpa*, 9. *C. reticulata* var. Keprok Bangli, 10. *C. reticulata* var. Slayer Mangguh, 11. *C. nobilis* var. Mangguh, 12. *C. reticulata* var. Keprok Mangguh, 13. *C. reticulata* var. Keprok Besakih.

92°C for 30 seconds with one rotation, denaturation at 92°C for 60 seconds, annealing at 60°C for 30 seconds, Elongation at 72°C for 90 seconds with 40 turns. Extension at 72°C for 90 seconds with one rotation [3]. PCR products were examined by electrophoresis in agarose gel TA 1% (w/v) and then sequencing DNA fragments CVPD^r. The sequencing process uses only primers *forward* (1F): GACTAGGTGGTAATACTACTTTT and primers *reverse* (1R): CCTTTTGGTCTATCTTTACTTA. PCR analysis results are presented in figures 3,4.

PCR analysis of DNA isolated from samples of several resistant and susceptible citrus plants showed that from 27 citrus plants samples of 25 samples contained CVPD^r (resistance genes), this was evidenced by their presence at 841 bp. The primary sequence used is the primer for CVPD^r detection. In figure 3a sample of the Siam Petang (*C. nobilis* var. Petang) not detected in line 4 (four), this proves that the sample of the plant did not have a resistance gene, it is likely that the sample used had a severe CVPD disease and was not detected by using the primary resistance gene.

In another PCR analysis that is in figure 4 shows that Kemuning (*M. peniculata*) on line 6 was not detected so there was no CVPD^r gene. This is because *M. peniculata* is a host plant for fleas. CVPD infectious disease insects will be more active at high temperatures (lowlands) compared to low temperatures (highlands). From the results of this study found the presence of CVPD^r gene in all types of citrus both vulnerable and resistant such as Tangerines, Siem citrus, Selayar citrus, Balinese citrus, Kingkit citrus and Seedless Lime citrus in all samples.

Polymorphism of DNA Fragments of CVPD^r genes

In addition, 25 of 27 citrus plants sample from the study showed that the sample contained DNA fragments of CVPD^r, susceptible to CVPD disease. The results of this study indicate that the CVPD^r DNA fragment in this citrus species or varieties does not work well and can be caused by mutations or resistance to CVPD disease needed by other genes. Therefore, we study the CVPD^r DNA fragment polymorphism. The DNA sequence DNA fragment CVPD^r is then performed and the results are shown in table 2.

Results showed a CVPD^r gene polymorphism between Tangerines citrus, Siam citrus, Selayar citrus, *T. trifolia*, Seedless Lime citrus, and all the citrus that are prone to CVPD. The occurrence of polymorphism in a gene, indicates the existence of some differences in DNA sequence in citrus plants sampled with different or similar species. The difference in DNA sequence is caused by the occurrence of deletions, insertions, recombination, low random marriage and selection in the population [6]. So far, through library research there has not been found a research report on the polymorphism of the CVPD^r gene fragment in citrus plants in Indonesia. In this study the CVPD^r gene showed that the target band was detected positively in 25 samples from 27 samples studied

at 841 bp. The electrophoresis method is a general method that can be applied to show polymorphisms of a large number of enzymes. With this method, it can be found changes in the structure of enzyme molecules that cause changes in the molecular electrical charge [7].

Figure 5 shows the phylogeny of several species of citrus plants in Bali. The phylogenetic tree is a logical approach to show the evolutionary relationship between organisms. Phylogenetics is a model to represent the relationship between ancestral organisms, molecular sequences or both. One of the goals of phylogenetic compilation is to precisely construct relationships between organisms and estimate differences that occur from an ancestor to its offspring. Phylogenetics can analyzed the changes that occur in the evolution of different organisms. Based on the analysis, sequences that have closeness can be identified by occupying branches next to the tree.

The results of this study indicate that there are many DNA polymorphisms among samples in the CVPD^r DNA fragment. Phylogenetic trees derived from sample polymorphisms show that Kingkit citrus (*T. trifoliata* var. Seedless) and Lime citrus (*C. aurantifolia*) are located in the same cluster, while other samples are in different clusters. These results indicate that DNA polymorphisms in CVPD^r DNA fragments cause differences in the resistance of citrus plants to CVPD disease. Polymorphism is the presence of more than one allele at a genetic locus with an allele frequency rarely more than one percent in the population. Gene polymorphisms occur due to changes in the composition of the nucleotides of genes. Changes in gene composition are influenced by several factors such as natural or artificial selection, mating and mutation. These changes can affect an organism's phenotypic changes [8].

Kinship and evolutionary relationships between organisms can be described through phylogenetic trees. The purpose of studying phylogenetic trees is to reconstruct *genealogical ties* (genealogies) between organisms and determine the evolutionary time of an organism. Phylogenetic tree is a graph consisting of branches or nodes. Two adjacent nodes are connected by one branch. Nodes represent taxonomic units, while branches represent relationships between taxonomic units. Branching patterns on phylogenetic trees are called topologies. The length of a branch represents the difference in that branch. Taxonomic units can be species, populations, individuals or genes [9].

Phylogenetic trees can be reconstructed using several statistical methods. One of them is the statistical method that is commonly used, namely *Bayesian methods*. *Bayesian method* is a phylogenetic tree reconstruction method that uses *optimality criteria* (optimal standard). The method has the same concept as the *likelihood method*, which uses *probability distribution of trees* to find the most appropriate phylogenetic tree from the data sequences used. The position

Table 2: CVPD^r gene polymorphism in some citrus plants in Bali.

No.	Size of bases (bp)	Bases Polymorphism	Isolate Sample
1	117	TCA TCT	CnKin
2	145-147	GAG TAA	Tt, CavarSeed, CnBll, CnPc, CrvarSlayBll, CnPy, CnTbn, CrvarKepMang, CrvarKepBs.
3	149	CTT CGT	CnKin
4	166-167	TGG CAG	CgKin, CgBs
5	181	AAA GAA	CgKin, CgBd
6	196	CAA TAA	CnKin
7	247	GCG AGG	CnKin
8	264	CAGCAT	CrvarSlayKin, CrvarKepGr, CrvarSlayMang, CrvarKepPet
9	269	TAT TGT	CnKin, CnBd, CgKin, CgBd, CgBs
10	275	AAT ACT	CnKin, CnBd, CgBs,
11	278	GAC G · C	CnKin
12	298	AGC GGC	CnBd
13	315	TAG TAT	CnBd, CnKin, CgBd, CgBs
14	336	AAA AAG	CnKin
15	344	GTT GCT	CnKin
16	347	TAT TCT	CrvarSlayKin, CrvarKep, CrvarSlayMang, CrvarKepPet
17	352	AAT CAT	CnKin
18	400-424	AGACTTTGTAGGCATAAATGCTAT	Only CnKin mutated
19	462	TTT TTG	CgBd, CrvarKepBgl
20	478	AAA CAA	CgBs
21	490	CAG TAG	CgKin
22	503-504	TTG AAG	CnKin, CnBd, CgKin, CgBd, CnBll,
23	527	GTT CTT	CnBd,
24	538	ATT ATG	CnKin, CnBd, CgKin, CgBd, CnBll
25	575	TAA CAA	CnKin
26	580-841	NNN NN	CrvarKepGr
27	585	TCT TTT	CnKin, CnKin, CnKin, CnKin, CnKinNNN NN CNPC, CnTbn, CnBd
28	591	TGG TAG	CnKin, CnBd, CgBd, CgKin, CgBs
29	597	ACC ATC	CnKin, CnBd, CgBd, CgKin, CgBs
30	631	TTC TTA	CnKin
31	633	ATA AGA	CnKin, CrvarSlayKin, CnBd, CgKin, CgBd, CrvarKepMang, CrvarKepBs, CgBs, CrvarKepBgl
32	652	ATG ATC	CrvarKepBgl, CgBs
33	663	AAA ACA	CnKin, CnBd, CgKin, CgBd, CgBs
34	672	TGT TTT	CgBs
35	680 bp	A · T ATT	CrvarSlayMang
	680 bp	A · T AAT	CnBll
	680-841	Bases there is a difference with another sample	CrvarSlayMang
36	694	TTC TTG	CnBd, CgKin, CgBd
37	706	AA · AAC	CnTbn
38	709	GTC GTT	CgBs, CnKin
39	714	TTA TCA	CnKin
40	724	ATA ATC	CgKin, CgBd
41	747	ATA AGA	CnBd
42	783	ATA AGA	CnBd
43	801	T · A TTT	CnKin

Note: CnKin = *C. nobilis* var. Kintamani, Tt = *T. trifoliata*, CavarSeed = *C. aurantifolia* var. Seedles, CnBll = *C. nobilis* var. Buleleng, CnPc = *C. nobilis* var. Pecatu, Crvar.SlayBll = *C. reticulata* var. Slayer Buleleng, CnPay = *C. nobilis* var. Payangan, CnTab = *C. nobilis* var. Tabanan, CrvarKepMang = *C. reticulata* var. Keprok Mangguh, CrKepBs = *C. reticulata* var. Keprok Besakih, CrvarSlayMng = *C. reticulata* var. Slayer Mangguh, Cauran = *C. aurantifolia*, Cambly = *C. amblycarpa*, CgKin = *C. grandis* C. L. Kintamani, CgBs = *C. grandis* L. var. Besakih, CgBd = *C. grandis* L. var. Bedugul, CrvarSlayKin = *C. reticulata* var. Slayer Kintamani, CrvarKepGr = *C. reticulata* var. Keprok Gianyar, CrvarKepPet = *C. reticulata* var. Keprok Petang, CnBd = *C. nobilis* var. Bedugul, CnGr = *C. nobilis* var. Gianyar, CrvarKepKr = *C. reticulata* var. Keprok Karangasem, CrvarBgl = *C. reticulata* var. Keprok Bangli, CnMang = *C. nobilis* var. Mangguh, CnDps = *C. nobilis* var. Denpasar

of each sequence in *aligned sequences* is called *character*, while nucleotides are called “states” in the *Bayesian methods*. The position of all *characters* is analyzed independently or separately from each other, so that each *alignment column* is assumed to be an independent realization in the evolutionary process. Bayesian methods allow researchers to determine their own models such as the model parameter substitution,

long branches, and tree topology [10], as shown in figure 5.

Conclusion

There are fragment DNA polymorphism CVPD^r on citrus that vulnerable and citrus plants that are resistant or tolerant to CVPD disease. PCR test showed *T. trifoliata* and *C. aurantifolia* var. Seedless are resistant to CVPD, and Phylogenically are in the

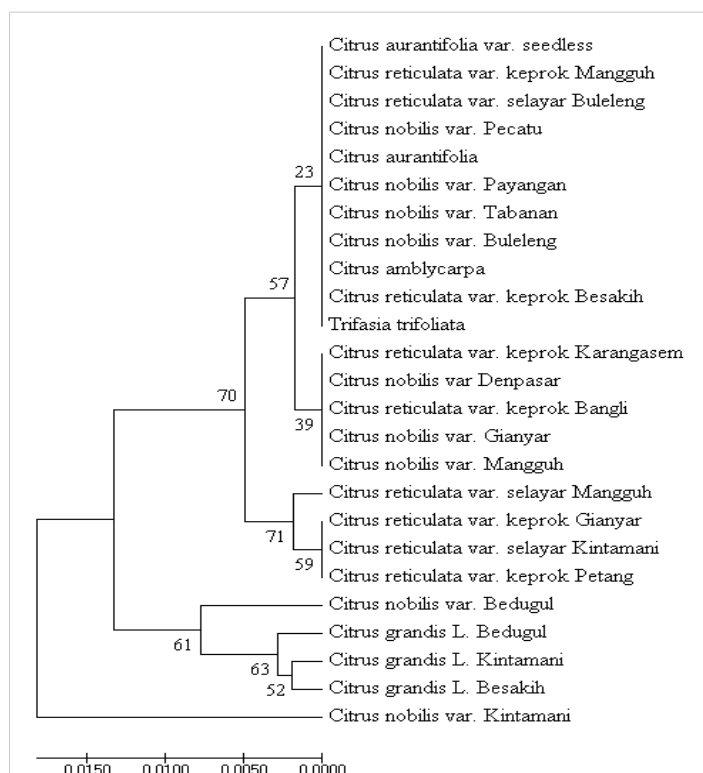


Figure 5: Phylogenetic tree is the result of UPGMA analysis by Bootstrapping various types of citrus plants in several regions in Bali based on nucleotide sequences. The length of each pair of the branches above shows the distance between each pair.

same group as *C. nobilis* var. Buleleng, *C. nobilis* var. Tabanan, *C. nobilis* var. Payangan, *C. nobilis* var. Pecatu, *C. reticulata* var. Selayar Buleleng, *C. reticulata* var. Keprok Mangguh, *C. reticulata* var. Keprok Besakih, and *C. amblycarpa*. On the other hand, citrus plants that are sensitive to CVPD are in a different group. There are two types of citrus plants not containing CVPD^r DNA fragments, namely *C. nobilis* var. Petang and *M. paniculata* L.

CVPD^r DNA fragments indicated have an important role in the mechanism of resistance of citrus plants to CVPD disease.

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