GENETICS DIVERSITY ANALYSIS OF SIX DIFFERENCE VARIETIES OF PUMMELO (*Citrus maxima* (Burm.)Merr.) FOR RESISTANCE TO HUANGLONGBING USING RAPD MARKERS

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INTISARI

Enam kultivar Pummelo : Raja, Ratu, Pangkajahe Merah, Pangkajahe Putih, Nambangan, dan Magetan di inokulasi dengan *Candidatus L. asiaticus* untuk mengetahui keragaman tanggapan ketahanan terhadap Huanglongbing. Keragaman dinilai berdasarkan intensitas penyakit, konformasi molekuler *Candidatus L. asiaticus* pada jaringan tanaman, dan keragaman molekuler pummel dengan analisis RAPD. Pangkahjahe Putih dinilai resisten terhadap HLB karena memiliki intensitas penyakit 0% dan hasil negatif dalam konformasi molekul *Candidatus L. asiaticus*. Hasil analisis RAPD diperoleh bahwa 865 bp merupakan fragmen yang dapat dikaitkan dengan kerentanan terhadap HLB.

Kata Kunci : Pummelo, keragaman, ketahanan, Huanglongbing, RAPD

ABSTRACT

Six Pummelo cultivars : Raja, Ratu, Pangkajahe Merah, Pangkajahe Putih, Nambangan, and Magetan were inoculated with *Candidatus L. asiaticus* to assess variability on resistance to Huanglongbing. Diversity was observed based on disease intensity, molecular confirmation of *Candidatus L.asiaticus* in plant tissues, and molecular variability of pummelo by RAPD analysis. Pangkajahe Putih was assessed being resistance to HLB by 0% in disease intensity and negative result in molecular confirmation of *Candidatus L. asiaticus*. RAPD analysis obtained an assessment that 865 bp fragment was associated with susceptible to HLB.

Keyword : Pummelo, variability, resistance, Huanglongbing, RAPD

INTRODUCTION

Citrus is believed to have originated from Asia region that have cultivated in Indonesia since hundreds years ago (BAPPENAS, 2000). Commercial citrus divided into four groups including orange, mandarin, pummelo and grapefruits, and common acid (Hodgson, 1967). Pummelo is the biggest citrus and be trusted as ancestor of grapefruit (*Citrus paradisi*). Pummelo (*Citrus maxima* (Burm.) Merr.) is one of the most cultivated citrus in Indonesia. The Chinese make various medicamens from the seeds, flowers, mature peel, and slices of this fruit by usually drying them up. It is used in threating cough, swellings, vomiting, indigestion, in removing phlegm and resolving toxins and hangover. The Malays eat the fruit to applied on swellings and ulcers. This fruit also is an excellent source of vitamin C and beta-carotene (Thulaja, 2003).

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One of the most destructing disease of citrus trees is Huanglongbing (HLB), formerly known as Citrus Greening, has destroyed an estimated 60 million trees in Africa and Asia (Timmer et al., 2003). This disease caused by *Liberibacter asiaticus* that transmitted by the Asian psyllid (*Diaphorina citri*). It has been infected many citrus cultivation area, such as Brazil, USA (Bové, 2006), China, and Taiwan (Tsai, et al. 2007). Citrus greening was firstly reported in Indonesia in 1940 that identified by the symptoms on citrus leaves of infected including reduced size, pale yellowing, and blotchy mottle (Sritamin, 2008). Those symptoms also were seen in *Citrus*. Spp., including pummelo.

In the past decade, the incidence of HLB in pummelo has been increasing along with increased pummelo production. Most citrus cultivars, except pummelo, were susceptible to the Asian form of HLB before 1971. However, pummelo became infected by a new HLB strain in Taiwan, China, and Southeast Asia in 1970s. The pummelo cultivars grown in Philippines, Malaysia, Southern China, Vietnam, Thailand, Indonesia, Sri Lanka, Bangladesh, and Cambodia have become susceptible to HLB in recent decades. It is caused by a nonculturable fastidious bacteria inhabiting the sieve tube and is referred to as *Candidatus Liberibacter*. It was assumed that the change of host range may have been due to the evolution of HLB strain in pathogenicity (Tsai, et al. 2007; Deng, et al. 2008).

Martin et al. (1991) have described an efficient method based on the RAPD technique to isolate DNA segments linked to certain traits. Ling et al. (2000) found RAPD and RGC (Resistance Gene Candidate) markers linked to a citrus nematode resistance region (designated *Tyr 1*) in a *Citrus-Poncirus* backcross population.

Localized linkage maps were constructed for the citrus tristeza virus resistance gene (*Ctv*) from *P. trifoliata* with RAPD, RFLP (Fang et,al. 1998; Mestre et, al. 1997), and SCAR (Deng et, al. 1997).

The objective of this research were to evaluate six different varieties of pummelo for their resistance to HLB, to ascertain the disease resistance, and to assess RAPD markers associated with gene(s) for susceptible to HLB.

MATERIALS AND METHODS

From Dec. 2009 to July 2010, ten of each six different accession of pummelo were inoculated with *L. asiaticus* and evaluated for its reaction to HLB (Table 1).in august 2010 three of each accessions were selected for RAPD analysis.

Isolation of total genomic DNA for RAPD analysis was carried out based on Karsinah (2002) with several modifications. DNA was quantified by DNA quantification processor GeneQuant 1300.

5 random 10-mer primers (A-02, E-14, K-04, N-16, W-19) that have polymorphic bands was selected from 7 primers (Operon Technologies Inc, USA). Each of 10 µL primer reaction mixture contained 2,5 µL DNA genome (2,5 ng/µL), 5 µL PCR mix *Go taq Green*, 2,25 µL *Nuclease-Free Water*, and 0,25 µL primer. The amplification were conduce according to Karsinah, et al (2002), denaturation for 5 min in 95C; followed.

by 45 cycles of denaturation at 95C in 30 sec, annealing at 39C for 45 sec, extension at 72C in 1 min, and final extension at 72C in 5 min.

The amplification products were separated on 1,75% agarose gels prepared with 1xTBE and poured onto a horizontal gel tray (BioRad), stained in ethidium bromide solution and visualized by UV transilluminator (Optima UVT 200). Gels were recorded using digital camera.

Photo of gels were analyzed using SequentiX – GelQuest 3.0.2 version. Each gels analyzed by the scoring of presence (1) and absence (0) of polymorphic bands in the individual lane. Simple matching coefficients and un-weighted pair-group method with arithmetic averages (UPGMA) were used to build phylogenetic tree using SequentiX-ClusterVis 1.6.0 version.

RESULTS AND DISCUSSIONS

Table 1. Reaction of six pummelo accession to HLB

No.	Accession	Origin	Disease Intensity (%)[1]	HLB-PCR test
1.	Pangkajahe Putih	North Sulawesi, INA	0,00 b	-
2.	Magetan	East Java, INA	3,33 b	-
3.	Raja	West Sumatera, INA	6,88 b	-
4.	Pangkajahe Merah	North Sulawesi, INA	6,88 b	-
5.	Nambangan	East Java, INA	10,00 ab	+
6.	Ratu	West Sumatera, INA	29,98 a	+

[1] = data was analyzed with ANOVA and DMRT test. Difference of notation after score indicate a significance of variance using DMRT test.

Correlation between reaction to HLB and DNA fragments were analyzed with fisher's exact test. If any fragment(s) had a correlation then this fragment(s) was analyzed with Kendall-tau correlation coefficient to find out form of this correlation.

Table 1 shows percentage of disease intensity and HLB-PCR test of each pummelo accession. Nambangan and Ratu have positive result in HLB-PCR test and the most two highest disease intensity in same notation. It indicated there is no significance disease intensity between Nambangan and Ratu. Nambangan also had positive result in HLB-PCR test, but disease intensity was not significant with other accession. The other accession was had negative result in HLB-PCR test and significant disease intensity with Ratu. Meanwhile, Pangkajahe Putih shows with negative result in HLB-PCR test and disease intensity 0%.

Table 2. The 5 primers were used for RAPD analysis and the fragments revealed

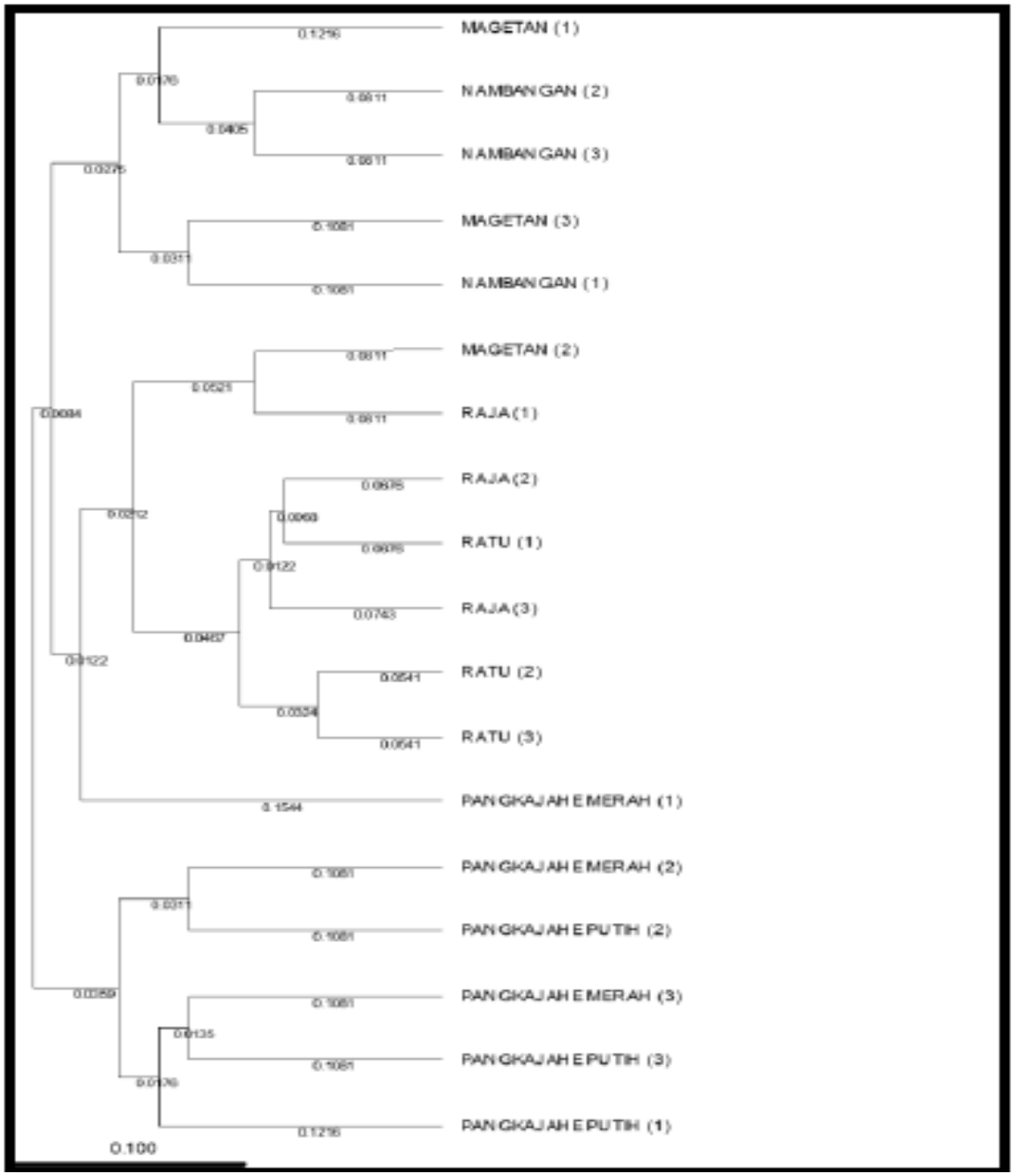
No.	Primer code	Sequences	Fragments		Total
			Monomorphic	Polymorphic	
1	OPA-02	TGCCGAGCTG	0	21	21
2	OPE-14	TGCGGCTGAG	0	7	7
3	OPK-04	CCGCCCAAAC	1	7	8
4	OPN-14	TCGTGCGGGT	0	19	19
5	OPW-19	CAAAGCGCTC	0	18	18
Total			1	72	73

Five primers were selected for the RAPD analysis based on the number of polymorphism created. The number of amplified fragments per primer varied from 7 to 21 (Table 2). Total fragments were amplified by 5 primers was 73 fragments with 72 polymorphism and 1 monomorphism. OPA-02 was amplified largest number of fragments among five primers with amount 21 fragments. The only one monomorphism fragments was amplified by OPK-04.

Genetic diversity of six pummelo accession was analyzed by SequenTix – ClusterVis with Simple Matching genetic distance method. This method was also used by Corraza-Nunes, et.al (2002) in assessment of genetic variability in grapefruits (*Citrus paradisi* Macf.) and pummelos (*Citrus maxima (Burm.)* Merr.) using RAPD and SSR markers.

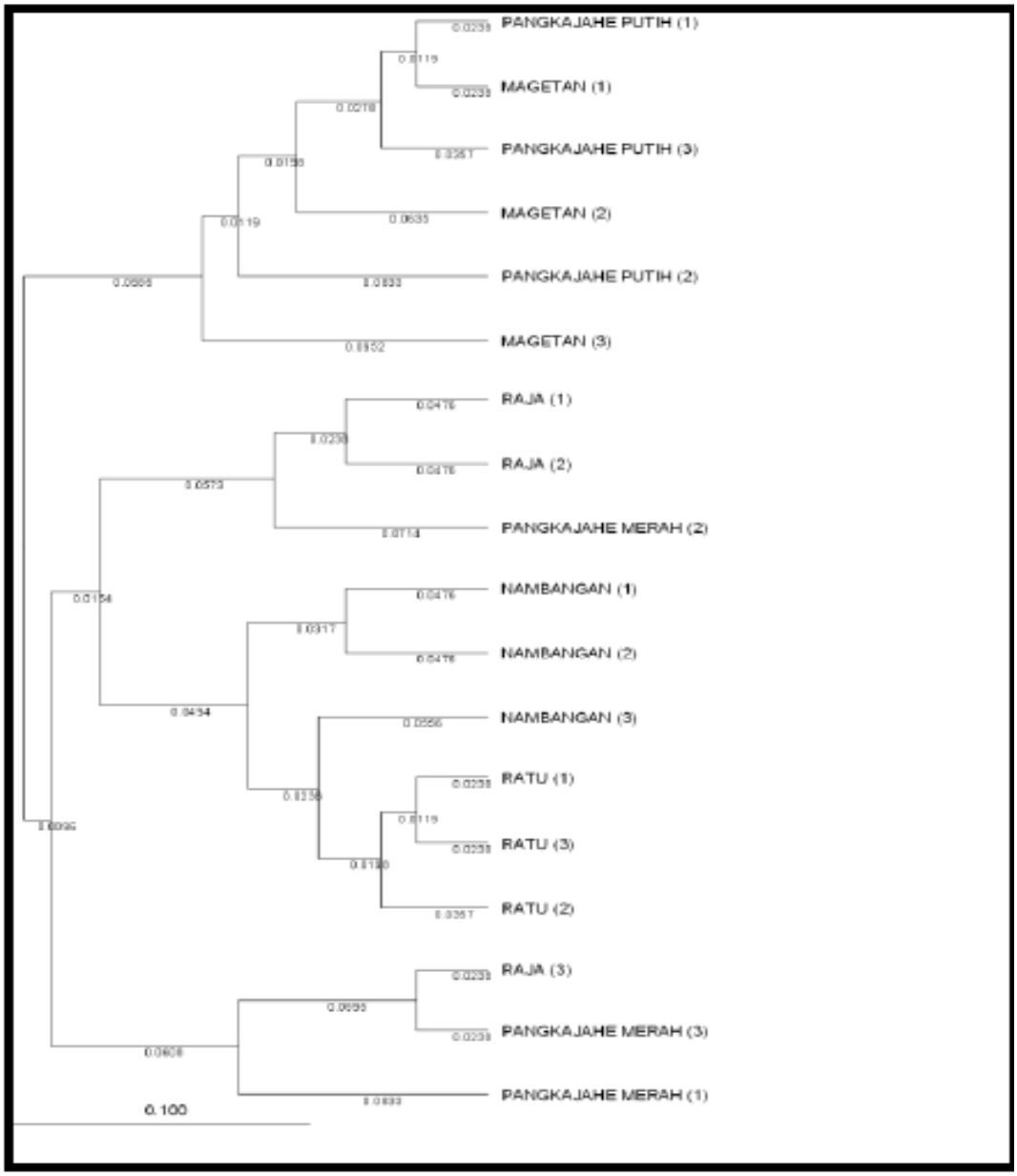
The analysis result shows that these 18 samples divided into 3 groups according to its origin. The first, Magetan and Nambangan were originated from East Java. The second, Raja and Ratu were from West Sumatera. The third,

Pangkajahe Merah and Pangkajahe Putih were from North Sulawesi. However, Magetan 1 and Pangkajahe Merah 2 were classified into the second group. This could be happen because of genetic variability of the two samples. In plant genetics perspective, pummelo was heterozygous, and RAPD markers detect dominant alleles. So this variation could be identified if the parental of these two pummelos was analyzed by RAPD markers.



(Fig. 1. Dendrogram of 6 pummelo accession, derived using the UPGMA grouping method based on simple matching similarity coefficients obtained RAPD analysis).

Generally, OPA-02 divided the samples into three groups. In further observed, this primer was discriminated the resistance and susceptive groups. Resistance group was consisted of ‘Pangkajahe Putih’ and ‘Magetan’ that have 0% and 3,333% disease intensity,also negative in PCR-HLB detection. Susceptive group was consisted of ‘Ratu’ and ‘Nambangan’ with disease intensity 29,98% and 10%, and positive in PCR-HLB detection.



(Fig. 2. Dendrogram of six pummelo accessions amplified with OPA-02, derived using the UPGMA grouping method based on simple matching similarity coefficients obtained RAPD analysis)

Correlation between DNA fragments and susceptible to HLB was proved by analyzing it with Fisher's exact test. The null hypothesis was there is no correlation between DNA fragments and susceptible to HLB ($p > \alpha=0,05$), and the alternative hypothesis was countered to it ($p < \alpha=0,05$). Among 21 fragments had been analyzing, Fisher's exact test found that there was any correlation between DNA fragments and susceptible to HLB in 865 bp. This hypothesis was proved by the size of $p = 0,043$. Fisher's exact test explain whether there is any correlation between DNA fragments and susceptible to HLB. However, this test didn't explain clearly about the similarity of these two parameters.

Kendall tau correlation was carried out to measure the association between DNA fragments and susceptible to HLB. The denominator is the total number of pairs, so the coefficient must be in the range $-1 \leq \tau \leq 1$. if the agreement between the two parameters are the same, the coefficient has value $0<\tau<1$, and $0>\tau>-1$ if the agreement is the reverse of the other (Conover, 1980). Kendall tau rank correlation found that the correlation between DNA fragments and susceptible to HLB were the same. This hypothesis was proved by the size of $\tau = 0,478$.

CONCLUSION

Data from disease intensity and PCR detection proved Pangkajahe Putih resistance to HLB. DNA analysis using RAPD markers divide six Pummelo cultivar into three groups based on its origin (i) West Sumatera, (ii) East Java, (iii) Sulawesi. OPA-02 primers was assessed 865 bp fragment linked to susceptible trait to HLB. Further research to ascertain fragment linked to HLB susceptible would be needed. Finally, the integration of genetic, ecological, and cost-benefit information seems mandatory to define a global Citrus HLB program.

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