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Genetic Resources of Rutaceous Plants against the Psyllid Vector Preference and Pathogen Transmission of Huanglongbing

By

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Introduction

Huanglonbing is the most important disease on citrus in Indonesia. The disease is caused by *candidatus* Liberibacter asiaticus and spread by the psyllid vector *Diaphorina citri* and by vegetative propagation. Almost all commercial citrus are susceptible to the disease, however, some citrus relative plants were reported to be the host of the psyllid but not the host of the pathogen or none of them as the host plants of the psyllid either the pathogen. Those plants reported as non host of the pathogen but may be as or may be not as the host plants of the psyllid are promising to be used as resistant genetic resources against the disease. This report is discussing the research on citrus relative trials for the preference of *D. citri* and the transmission of Liberibacter. Furthermore, the species of *Muraya paniculata* and *M. exotica* both as the host plants of the psyllid, however, only one of them was reported as the host plant of Liberibacter. Unfortunately, the characteristics of *M. paniculata* and *M. exotica* have been in doubtfully mix up, therefore the name of *M. paniculata* in a certain report is given for *M. exotica.* Therefore this report is also discussing the characteristic of *M. paniculata* compared to *M. exotica* morphologically and molecularly.

Materials and Methods

A. Green House Experiment of Citrus Relative Trial for the Preference of *D. citri*

Disease free of *D. citri* was obtained from cultures which were maintained on *Muraya paniculata* in the control temperature glass house (CT). CT temperature for rearing was set on 28°C and relative humidity set on 60%.

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Experiment was set up in choice mode with seven species of citrus relative plants and five replications. *Swinglea glutinosa, Aegle marmelos, Murraya paniculata*, *Murraya exotica, Limonia acidisima, Triphasia aurantifolia* and *Glycosmis pentaphylla* plants were used.

Plants were placed in the transparent plastic cage (63 cm diameter, 90 cm high), covered by nylon mesh on the top. Plants arrange randomly in circular style. 20 *D. citri* adults were released in the middle of the arena in each cage. The number of *D. citri* sit on each plant was recorded twice a day for seven days, at 10 am and 4 pm. *D. citri* found to be sitting on the plants were considered as feeding on the plants.

B. Filed Trial Experiment of Citrus Relative Plants for the Preference of *D. citri* and HLB Transmission

The number of 20 different plant species (or cultivars) of Rutaceae (Table 1) were planted on the land randomly with 16 replicate blocks. The distance between plants are 2 m and *C. reticulata* cv. Siem was planted as border plants between blocks. The experiment is conducted at low land of about 100 m also in the endemic area of HLB surrounded by several citrus orchards belongs to the local farmers. The cultivation of the plants was as a standard Siem mandarin cultivation by 2 applications of fertilizer a year of 5 kg organic fertilizer/plant and 50 g or ZA/plant in the first year.

Table 1. Name of live collection species of *Murraya* spp for morphological and molecular (DNA isolation) characterization.

No.	Name of species	Origin	Number of sample
1	*M. crenulata*	Bogor Botanical Garden	1
2	*M. crenulata*	Purwodadi Botanical Garden	1
3	*M. exotica*	Bogor and around	6
4	*M. exotica*	Yogyakarta and around	14
5	*M. exotica*	Purwodadi and around	5
6	*M. koenigii*	Bogor and around	8
7	*M. koenigii*	Yogyakarta and around	12
8	*M. koenigii*	Purwodadi and around	10
9	*M. paniculata*	Bogor and around	10
10	*M. paniculata*	Yogyakarta and around	10
11	*M. paniculata*	Purwodadi and around	5

Each plant in every block was observed for the psyllid population every fortnightly and one year observation has been conducted. The HLB like symptom was observed however PCR confirmation was conducted one year after planting.

C. Characterization of *Muraya* spp.

To support the experiment on citrus relative trial for the preference of *D. citri* and the transmission of HLB characterization of *Muraya* spp. was conducted. Morphological and molecular characterizations were conducted on several samples originated from different localities in Java.

-C.1. Morphological characterization

In morphological study, parameters observed are characters such as:

a. shape (low scrub; high scrub, low tree; high plant)
b. stem (ramification; stem surface; hair at stem; stem color)
c. leaves (type of multiple leaves; maximum length of multiple leaves; maximum amount of leaflet; pair of leaflet; surface of petiole; shape of leaflet; sessile leaflet; clear hole of oil gland node; amount of oil gland per 1x1 cm; hair in oil gland; amount of hair in oil gland; hair in leaf vein; leaf edge; leaf end; leaf base; shape of leaf base; ventral; dorsal; midrib; hair in midrib; leaf vein; lateral bone; leaf thickness; petiole; hair in vein)
d. flower (type of inflorescent, amount of flower; inflorescent position; firstly blossom; pistil growth, flower size; sepal size; position of sepal; petal; shape of petal; size of petal; position of petal; size of outer filament; size of inner filament)
e. fruit (size of fruit; color of ripe fruit; fruit peel; shape of fruit)
f. seed (shape; color; color of aril; position of cotyledon at growing)
g. leaf anatomy (amount of palisade parenchyma; dense of palisade parenchyma)
h. flower anatomy (position of placenta)

-C.2. Molecular characterization

DNA was isolated from plants obtained from living collections located at Bogor (06 36' S, 106 48' E), Purwodadi (07 07' S, 110 55' E), and Yogyakarta (07 49' S, 110 22' E) using the CTAB method of Komar (1999). DNA amplification was based on the method of Karsinah (2002). The amplification was conducted in 15 L of solution consisting of 7.5 L of PCR master mix from Microzone Ltd, 1.5 L of primer 15 pmol, 4.5 L of MilliQ water, and 1.5 L of template DNA using My Cyber thermocycler (BioRad). The primers were used for amplification were OPU-3, 6, and 7, and OPN-16 and OPW-19. An initial denaturation was carried out at 95°C for 5 minutes, followed by 45 cycles of denaturation at 94°C for 30 s,

annealing at 36℃ for 30 s, and extending at 72℃ for 80s. A last cycle with extension at 72℃ for 10 min was then performed. Electrophoresis was performed at 100 V for 25~30 min in a 1.0 % agarose gel in which ethidium bromide had been added. The amplified DNA fragment patterns were visualized using a UV transilluminator and photographs taken.

D. HLB infection on Muraya spp.

Natural infection of HLB on Muraya was observed elsewhere and confirmed using PCR analysis. Inoculation of Muraya seedlings using *D. citri* was conducted in the greenhouse. Seedlings at the age of 5~6 weeks were invested with infectious D. citri obtained from endemic area of citrus HLB then observed for the symptom development and PCR confirmation.

Results

A. Green House experiment of citrus relative trial for the preference of *D. citri.*

The number of *D. citri* adult stay on citrus relatives is showed in Figure 1. *D. citri* feed on *Aegle marmelos* was constantly highest than others. On *Glycosmis pentaphylla* the number of D. *citri* tend to be decreased, but on *Limonia acidisima* tend to be increased.

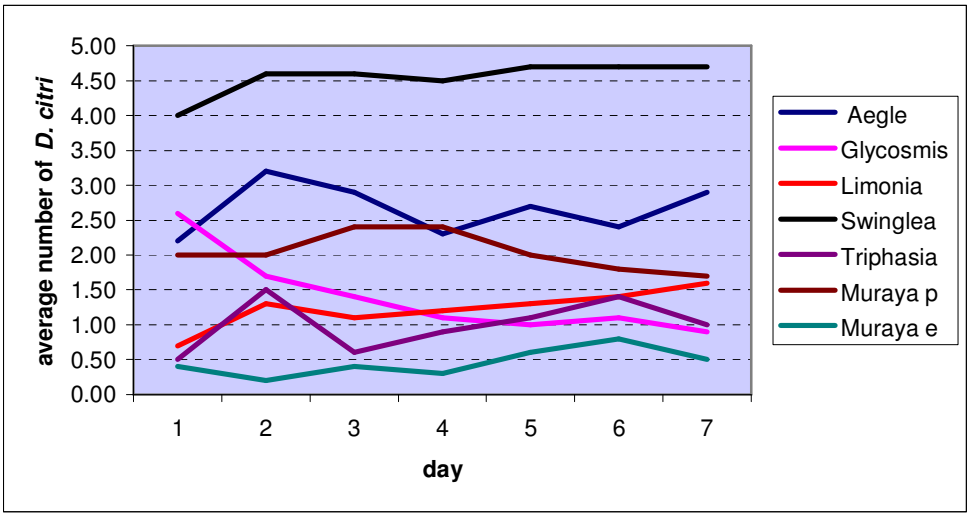

Figure 1. The fluctuation number of *D. citri* adult stay on citrus relatives which is considered as a feeding site

D. citri showed significantly ($P < 0.001$) highest preference to feed on *Swinglea glutinosa*. The least interest for feeding was on the *Murraya exotica* and no plants were found to be rejected by *D. citri* for feeding, as showed in Figure 2.

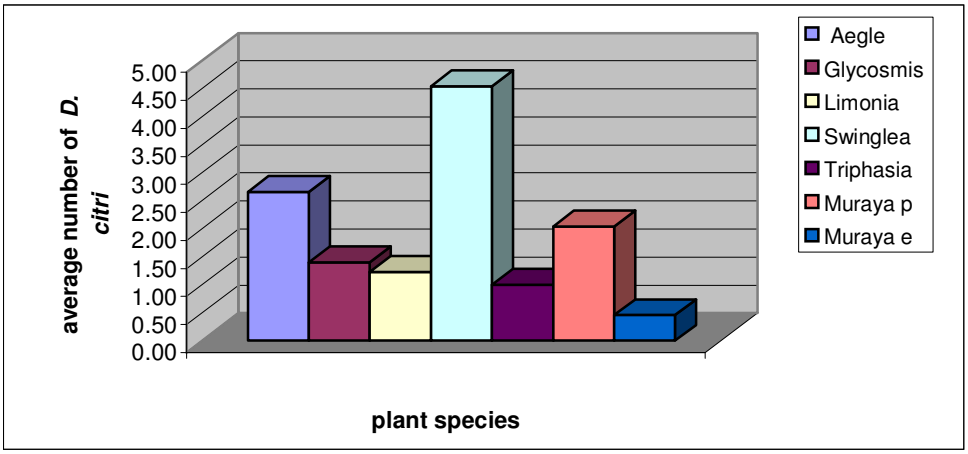

Figure 2. The average number of *D. citri* feeding on citrus relatives

B. Filed trial experiment of Citrus relative plants for the preference of *D. citri* and HLB transmission

From 20 species/cv of Rutaceae, the species/cv which were not visited by *D. citri* in year 2006 included *Aegle marmelos, Limonia acidisima*, *Feroniela lucida*, *Glycosmis pentaphylla*, *Clausena harmandiana,C. lancium,C. reticulata* var Grabag, and *C. r.* var. Siem. Those two varieties of *C. reticulata* were probably escaped from *D. citri* visit due to the same or very similar to those border plants which are *C. r.* var. Siem. There were 12 species/cv which were visited by *D. citri* in the field trial of citrus relative plants tested. The data on the population of *D. citri* on those species/cv is showed in Figure 3. The *D. citri* population on those species/cv of citrus relatives were not related to the transmission of HLB as PCR confirmation was conducted with no positive results was found. Further observation is being continued for the following years both for the population dynamic of *D. citri* and HLB transmission.

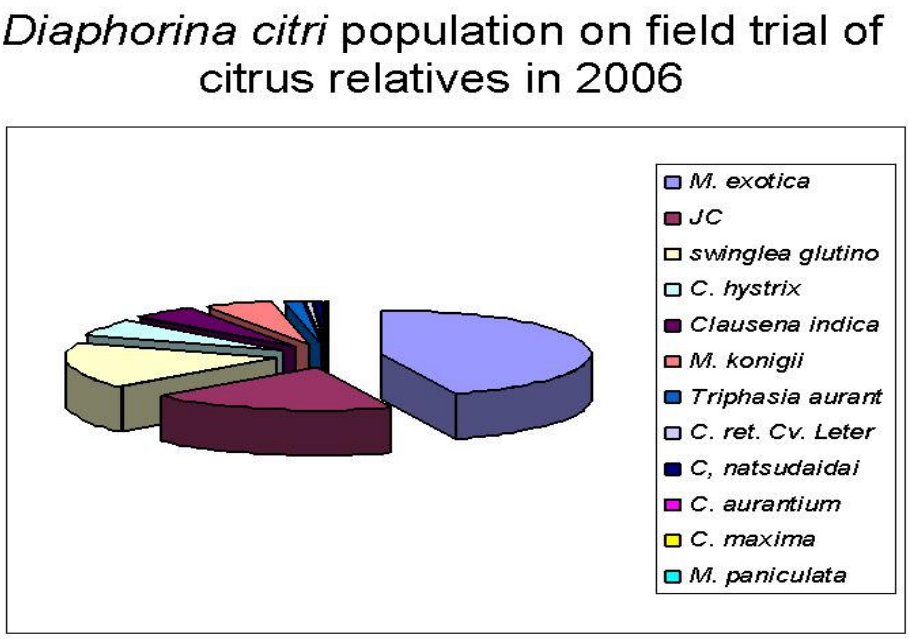

Figure 3. *Diaphorina citri* populations on field trial of citrus relative in 2006

C. Morphological Characterization of *Muraya* spp.

-C1. Characterizations

M. koenigii is separated from three other species of *Murraya* because the species has spread leaf sessility. Leaf sessility is fixed character because it is not influenced by outside factor. *M. crenulata* is distinguished to *M. exotica* and *M. paniculata* based on hair structure present in surfaces of stem, branch, leaf and petiole as well as in oil gland. Existence of hair indicates genetic nature, because the hairs have functioned as protector, reducing evaporation. It is also for even panicle inflorescent shape and its position only in stem end. Character that is also used to distinguish *M. crenulata* agrees with character used in Hartley (1993)'s description.

Separation of *M. exotica* and *M. paniculata* is due to leaf shape, flower growth, fruit shape and seed shape. The characters are nature based on genetic nature. Use of the characters supports Stone (1985) and Uji (1994), although Backer and Bakhuizen vd Brink (1965) and Swingle (1967) stated that *M. exotica* is synonym of *M. paniculata*, and Huang stated it as variety (*M. paniculata* var *exotica)*. Therefore, it is clear that in morphological manner *M. exotica* and *M. paniculata* can be distinguished as different species. To get more certainty, quantitative analysis using numeric taxonomical approach was done.

-C2. Molecular Characteristics

RAPD analysis using primers OPM-16 and OPW-19 produced DNA banding patterns that support the separation of *M. exotica* and *M. paniculata* into two species. However, as the origins of the material in Indonesia are unknown, the possibility exists that it may have come from one original introduction. Therefore, further RAPD analysis was conducted using primers OPU-3, OPU-6 and OPU-7. Amplification with these primers gave variable results and there was no consistency in the banding patterns produced from DNA extracted from different accessions of the two species. Therefore, the accessions of the two species all appear to have come from different sources and supports the separation of the two species based on data using OPM-16 and OPW-19.

D. HLB infection on *Muraya* spp.

HLB infection naturally was found on *M. exotica* in Denpasar Bali and PCR confirmation was positively observed. Inoculation on *Muraya exotica* was resulted on the symptom development and positive PCR confirmation but not on *M. paniculata*.

Discussion

Citrus greening is very severe disease and infecting mostly all commercial citrus in Indonesia and other countries. Genetic resources for resistance against the disease are needed. Therefore the experiments of citrus trial for the preference of HLB psyllid vector *D. citri* and the transmission of HLB are needed to be further conducted. The experimental results discussed in this paper are that from 20 species/cv of Aurantioidea plants, the psyllid in the field has preference on certain species or citrus cultivars (Figure 3). However, the species which were not visited by the psyllid in the field experiments were visited in the green house experiment using cages. Therefore, it was understood that psyllid landing on a certain species may be having several purposes which were only temporary visit, for feeding and laying eggs or maybe for protection of avoiding from hazardous environment. The most important reason for the psyllid visiting the species is when they feed on them because it will be depositing the HLB pathogen inoculums. The successful of transmission naturally and artificially by psyllid was on *M. exotica*, and in the field. We found the highest population was found on that species. Furthermore, the report from Brazil HLB suggested that several times infected Muraya were found, which they claim as *M. paniculata* but on our rough description we know that the plant was *M. exotica* (Silvio Lopes, personnel communication). Further collaboration to identify the species of Muraya naturally infected by HLB in Brazile and the genetic diversity of the pathogen is progress.

Further research may lead to the psyllid behavior visiting citrus relative plants related to feeding purposes and the durability of infection of HLB in the citrus relative plants. The resistance gene may obtain from the plants which the psyllid feed on and transmission the pathogen; however the disease is inhibited to develop further.

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