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Short Communication

Isolation and characterization of two entomopathogenic fungi attacking *Diaphorina citri* (Homoptera, Psylloidea) in Indonesia

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In an attempt to suppress the propagation of citrus greening disease in Indonesia, we explored pathogens of *Diaphorina citri* which vectors the disease. At two orange orchards, many *D. citri* adults were found to be dead and covered with fungal mycelia. Two fungi, *Paecilomyces fumosoroseus* and *Hirsutella citriformis*, were consistently isolated from the infected insects. Molecular phylogeny of their 18S rDNA sequences showed that they belong to the ascomycetous clade of the Clavicipitales/ Hypocreales, which embraces many entomopathogenic fungi. When healthy adults of *D. citri* were inoculated with conidia of the *P. fumosoroseus*, the insects died within 6 d.

Key Words—biological control; citrus greening disease; *Diaphorina citri*; *Hirsutella citriformis*; *Paecilomyces fumosoroseus*.

Citrus greening is one of the most important diseases of oranges in Asia and Africa. Since infected trees are severely damaged, orange production is often significantly reduced where citrus greening is widespread. The leaves of affected plants become yellowish, leathery, upright on the twigs, and the phloem tissues collapse (da Graça, 1991). The causative agent of citrus greening was identified as uncultured, gram-negative, walled, and phloem-limited bacteria, "*Candidatus* Liberobacter asiaticum" in Asia and "*Candidatus* L. africanum" in Africa, belonging to the α subdivision of the *Proteobacteria* (Jagoueix et al., 1994). The disease is transmitted from infected to healthy plants by psyllids, *Diaphorina citri* in Asia and *Trioza erytreae* in Africa (McClean and Oberholzer, 1965; Capoor et al., 1967; Schwarz et al., 1970). Therefore, in order to control the citrus greening disease, it is important to control the psyllid vectors. In Indonesia, citrus greening is widespread throughout the archipelago, vectored by *D. citri*, and causes great damage to orange production (Tirtawidjaja, 1980). In orange orchards in central Java, we found that a number of dead *D. citri* were covered with mycelia, which prompted us to explore fungal pathogens controlling the population of *D. citri*.

Fungal infection rate of *D. citri* was monitored in two orange orchards, Balai Penyuluhan Pertanian (BPP) and Macanan, about 5 km distant from each other, located at Jatinom, Klaten district, central Java. At each location, 10 citrus trees, *Citrus nobilis*, were selected as monitoring sites. The dynamics of population density and fungal infection rate of *D. citri* were quantitatively assessed as follows. The leaves of each tree were observed for 5 min, during which individuals of *D. citri* found on the tree were scored as either nymphs, uninfected adults, or infected adults. The results from the 10 trees were summed to obtain data for specific dates at the location. Monitoring was conducted every two or three weeks, from 3 September 1998 to 11 February 1999 at BPP and from 11 February to 29 July 1999 at Macanan.

Figure 1 shows the dynamics of population size and fungal infection rate of *D. citri*. Nymphs and adults of *D. citri* were found throughout the research period, which indicates year-round reproduction under the tropical climate. At almost every time of monitoring, except two times at BPP and two times at Macanan, a considerable proportion of adults were dead and covered with fungal

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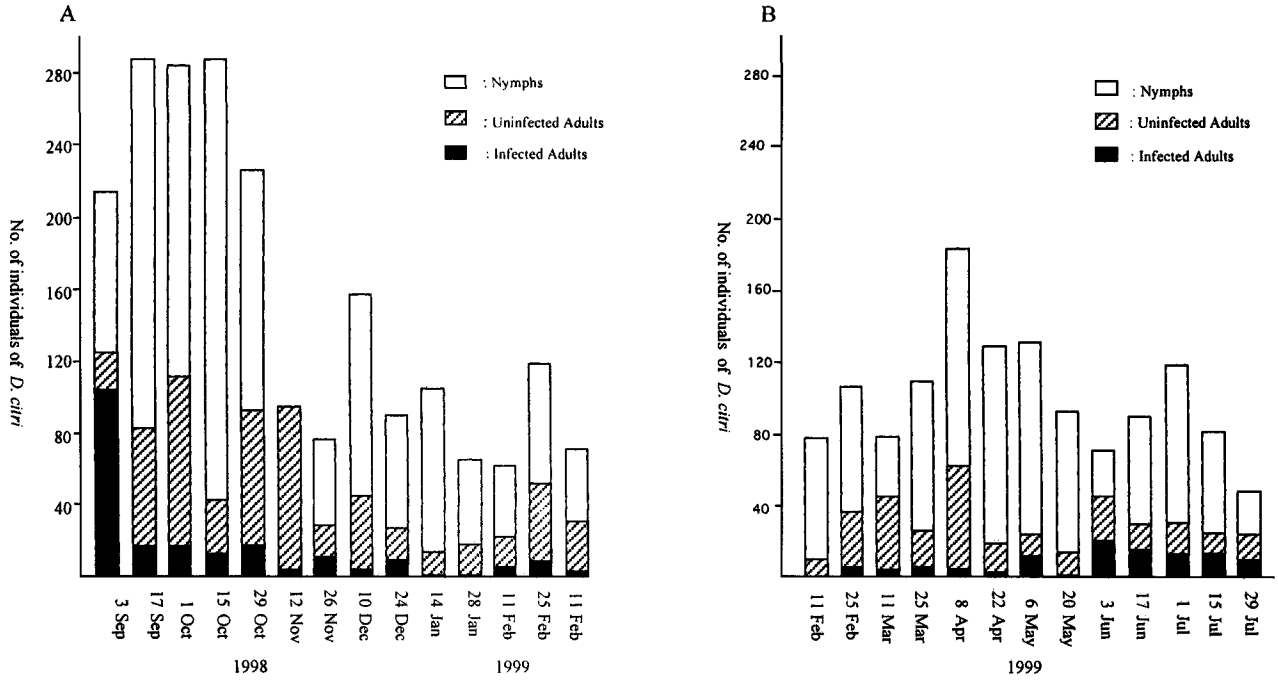

Fig. 1. Dynamics of population density and fungal infection rate of *D. citri* at two citrus orchards in Indonesia. (A) At BPP from 3rd Sep. 1998 to 11th Feb. 1999. (B) At Macanan from 11th Feb. 1999 to 29th Jul. 1999.

mycelia on the leaves. On the other hand, we could not find any nymphs covered with mycelia. Maximum infection rate in adults was 82.9% at BPP (Fig. 1A) and 52.2% at Macanan (Fig. 1B).

Infected *D. citri* adults were collected in the citrus orchards. They were washed several times with sterile water, and then mycelia, synnemata, or insect bodies were inoculated onto 1.5% agar plates of YMPD medium (3 g/l yeast extract, 3 g/l malt extract, 5 g/l polypeptone, 10 g/l dextrose, 0.1 U/ml penicillin) and cultured at room temperature. Mycelia growing out of the inocula were transferred to new plates several times to establish pure fungal strains. Single conidium isolation was also conducted to obtain pure isolates.

From the infected adults of *D. citri*, two types of fungi were consistently isolated, although other types of fungi, probably due to contamination, were sporadically obtained. One type formed a woolly colony, white in color, and grew fast. The other type formed a hard and elastic colony, with a pinkish or brownish hue, and grew slowly. The former produced abundant conidia on YMPD plate, while the latter produced only a few. Three strains of the former type, DCP03 and DCP04 from Macanan and DCP05 from BPP, were isolated. Two strains of the latter type, DCH04 from Macanan and DCH05 from BPP, were also established. The fungal strains are kept at the Plant Pathology Culture Collection, Faculty of Agriculture, Gadjah Mada University, Indonesia.

To identify the isolated fungal strains, their morphological characteristics were observed by light microscopy. The isolates were cultured on 1.5% agar plates of SDY (Sabouraud's dextrose medium with yeast extract) medium (10 g/l yeast extract, 10 g/l polypeptone, 20 g/l dextrose), and incubated at 25°C. Conidiophores and synnemata were mounted in lactophenol with or without cotton blue.

Strain DCP03: For 20 phialides measured, the size was 3.6–6.0 × 1.5–2.2 μm, mean 4.7±0.2 (SE) × 1.9±0.1 (SE) μm, and each had a globouse or elipsoidal portion tapering into a distinct neck, 0.3–0.6 μm wide. Conidia were cylindrical to fusiform, smooth-walled, hyaline, pale grayish pink color in a mass. For 20 conidia measured, the size was 2.3–3.5 × 1.1–1.7 μm, mean 2.9±0.1 (SE) × 1.4±0.1 (SE). These characteristics agreed with the description of *Paecilomyces fumosoroseus* (Wize) A. H. S. Brown & G. Smith (Samson, 1974). Strains DCP04 and DCP05 exhibited almost the same morphological characters (data not shown).

Strain DCH04: 3 wk after the inoculation, synnemata pale brown in color grew up from the inocula, which were placed in the center of plates. About 5 wk after the inoculation, a number of new synnemata grew from the periphery of the colonies. Sometimes these synnemata branched, with maximum lengths of about 5 mm. Phialides were flask-shaped, 5.1–9.4 × 3.5–4.7 μm, mean 7.2±0.2 (SE) × 3.9±0.1 μm (SE) in size (N=20), attenuated into a simple sterigma, 22.4–52.9 μm, mean 32.5±0.6 μm (SE) (N=20) in length, from which conidia were produced. Conidia were one-celled, hyaline, subfusiform, 6.4–7.6 × 2.1–2.8 μm, mean 6.7±0.1 (SE) × 2.4±0.1 (SE) (N=20), covered with mucilaginous substance and stuck together on the top of sterigmata. These morphological characters, together with the fact that this fungus originated from a hemipterous insect, agreed with the description of *Hirsutella citriformis*

Speare (Aoki, 1989). Strain DCH05 exhibited similar morphological characters (data not shown).

To determine the phylogenetic placement of these fungi and to confirm the morphological identification, 18S rDNA of strains DCP03 and DCH04 was analyzed. Fungal body was ground well into powder in a mortar in the presence of liquid nitrogen, from which DNA was extracted using the QIAamp tissue kit (QIAGEN). From the whole fungal DNA, almost entire length of 18S rDNA was amplified by PCR using primers NS1 [5'-GTAGTCATATGCTTGTCTC-3'] and FS2 [5'-TAGGNATTCCTCGTTGAAGA-3'] with TaKaRa LA Taq DNA polymerase (TAKARA) under the temperature profile of 94°C for 2 min, followed by 30 cycles of 94°C for 1 min, 50°C for 1 min and 70°C for 3 min, and final extension at 70°C for 2 min. The amplified product was purified

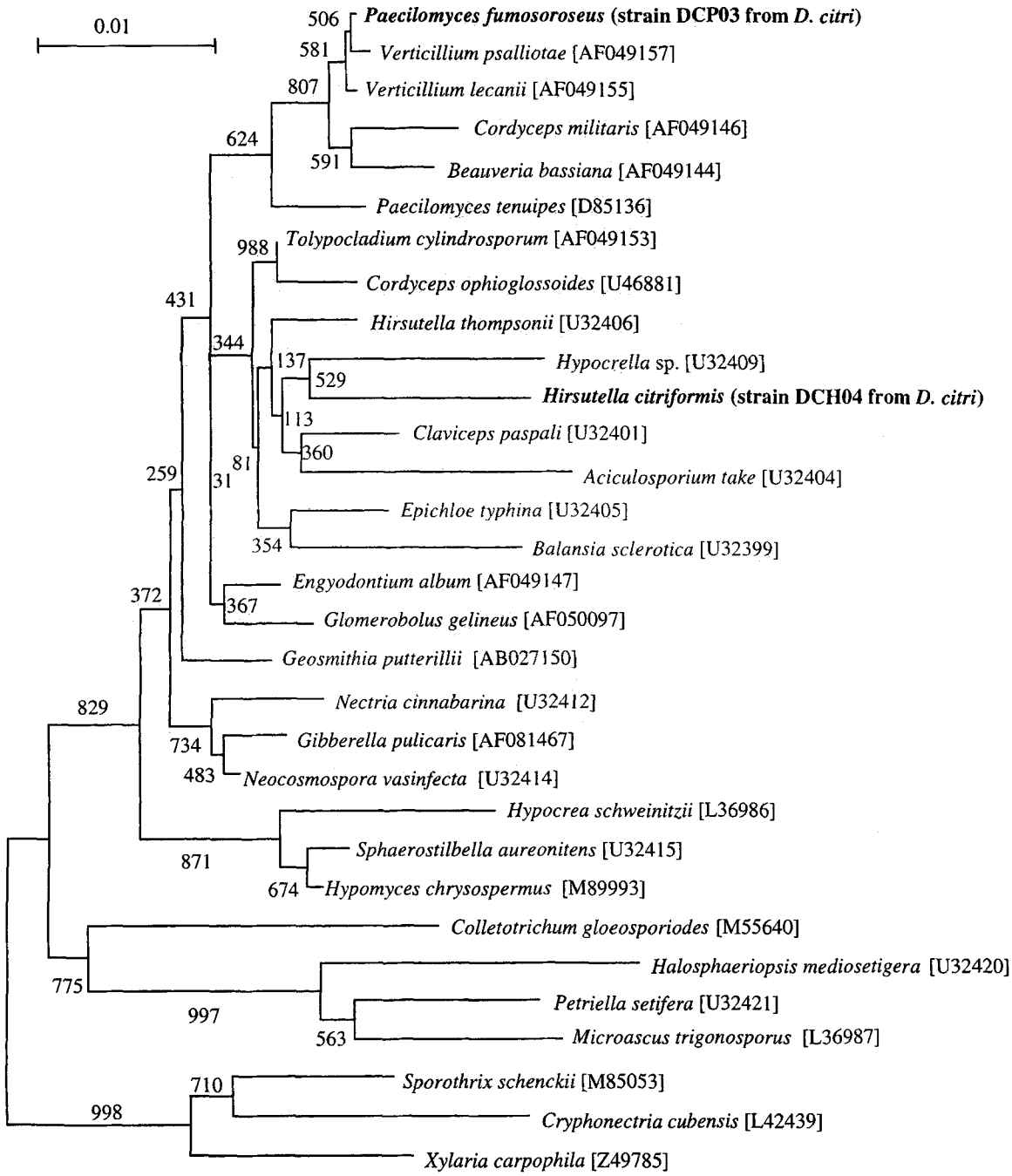

Fig. 2. Phylogenetic position of the strains DCP03 and DCH04, isolated from *D. citri*, in the Ascomycota. A total of 866 unambiguously aligned nucleotide sites of 18S rDNA were subjected to molecular phylogenetic analysis. A neighbor-joining tree is presented, whereas maximum parsimony analysis gave essentially the same result. Bootstrap values obtained with 1,000 resamplings are shown at the nodes. The numbers in brackets are accession numbers.

by Geneclean II kit (BIO 101 Inc.), and cloned with pT7 Blue vector (Novagen) and *Escherichia coli* strain DH5α competent cells. Plasmids containing the PCR products were isolated using QIAprep-Spin Miniprep Kit (QIAGEN), subjected to dye-terminator labelled cycle sequencing reaction with amplifying and internal primers using Big-Dye DNA Sequencing Kit (Perkin Elmer), and analyzed with ABI PRISM 377 DNA sequencer (Perkin Elmer). The sizes of the sequence determined were 1,520 for *P. fumosoroseus* and 1,520 for *H. citriformis*. The nucleotide sequences of their 18S rDNA were deposited in the DDBJ/EMBL/GenBank nucleotide sequence databases with accession numbers AB032475 and AB032476, respectively.

The sequences, together with 18S rDNA sequences of other fungi in the databases, were multiple-aligned using the program package Clustal X (Thompson et al., 1994). The final alignment was inspected and corrected manually. Ambiguously aligned nucleotide sites were excluded from the phylogenetic analysis. Nucleotide sites that included alignment gap(s) were also omitted from the aligned data set. The sequence alignment is available upon request from the corresponding author. Neighbor-joining trees (Saitou and Nei, 1987) were constructed with Kimura's two-parameter distance (Kimura, 1980) using Clustal X. Maximum parsimony trees were constructed using PAUP ver. 3.1.1 (Swofford, 1993). To assess the confidence of phylogenetic relationships, the bootstrap test (Felsenstein, 1985) was conducted with 1000 resamplings.

Figure 2 shows the phylogenetic placement of the *P. fumosoroseus* strain DCP03 and the *H. citriformis* strain DCH04 based on their 18S rDNA sequences. Phylogenetically, both isolates were obviously ascomycetous fungi in the clade of the Clavicipitales/Hypocreales. DCP03 clustered with entomopathogens such as *Verticillium psalliotae, V. lecanii, Cordyceps militaris, Beauveria bassiana* and *Paecilomyces tenuipes*. This result was concordant with the morphological identification of DCP03 as a *Paecilomyces* species. DCH04 clustered with entomo- and phyto-pathogens such as *Hypocrella* sp., *Hirsutella thompsonii, Claviceps paspali* and *Aciculosporium take*. This result also agreed with the morphological identification of DCH04 as a *Hirsutella* species. However, it should be noted that these groupings were statistically not significant because, among the members of the Hypocreales/Clavicipitales, their 18S rDNA sequences were too similar to each other to resolve their relationships.

Finally, in order to confirm that the fungal isolates could infect and kill *D. citri*, a preliminary pathogenicity test was conducted. Because the *Paecilomyces* strains produced abundant conidia on YMPD medium while the *Hirsutella* strains did not, strain DCP03 was used in the experiment. Inoculation was conducted on citrus seedlings with 3 or 4 leaves growing on sterilized soil in small plastic pots. Conidia formed on a well-grown plate were suspended in 0.02 M phosphate buffer (pH 7.2) containing 0.02% Tween 20. Density of conidia was measured under a microscope using a hemocytometer. The spore suspension (10^7–10^9 conidia/ml) was sprayed onto the seedlings and dried, to which 5 to 10 healthy adults of *D. citri* were introduced. The seedling pots were inspected daily to check the survival and fungal infection of the insects. For negative control, phosphate buffer alone was sprayed instead of the spore suspension.

Table 1. Artificial inoculation of healthy *D. citri* with conidia of *Paecilomyces fumosoroseus* (strain DCP03).

Inoculum[1] (conidia/ml)	10^9	10^8	10^7	Control
No. of insects tested	20	27	21	48
No. of insects infected[2]	7	15	4	0
Infection rate (%)	35.0	55.6	10.0	0.0

[1] The amount of suspension sprayed was not measured exactly but standardized by the number of pushes of the sprayer pump.
[2] After 6 d of incubation, the number of dead insects with mycelia was counted.

Table 1 represents the results of the inoculation test. Within 6 d, a considerable proportion of inoculated insects were dead, and mycelia were growing out of them. In contrast, no insects in control experiments showed such symptoms. Therefore, it was demonstrated that the *P. fumosoroseus* DCP03 strain had the ability to attack and kill *D. citri*.

In the present study, we isolated and morphologically characterized two fungi, *P. fumosoroseus* and *H. citriformis*, from adults of *D. citri* infected by fungal pathogens in Indonesia. Molecular phylogenetic analysis showed that they are placed in the ascomycetous fungal group Clavicipitales/ Hypocreales, which embraces many entomopathogens including *Beauveria, Cordyceps, Hirsutella, Hypocrella* and *Paecilomyces*. When healthy *D. citri* were inoculated with conidia of an isolate of *P. fumosoroseus*, the insects were infected and killed by the fungus. In conclusion, we obtained two entomopathogenic fungi that may be potentially useful for biological control of *D. citri* and citrus greening disease it vectors.

In Indonesia, citrus greening disease is widespread throughout the archipelago and causes enormous damage to orange production (Tirtawidjaja, 1980). Control of *D. citri*, the vector of the disease, is a very important agricultural problem in Indonesia. However, because Indonesia is a developing country, imported chemical pesticides are in general very expensive for the majority of farmers. Recent economic crises in Indonesia have made the situation much worse. Microbial pesticides are, therefore, a promising alternative in this country. Various types of microorganisms such as viruses, bacteria, fungi and protozoa have been utilized as microbial pesticides. Among them, it is known that humidity is particularly important for the survival and activity of fungal pesticides applied in the field (Burges, 1981; Baker and Dunn, 1991; Tanada and Kaya, 1993). For biological control of pest insects, fungal entomopathogens as reported in this study appear to be suitable for the social and natural conditions in Indonesia.

In addition to these entomopathogenic fungi, other

types of natural enemies controlling the population of *D. citri* should be explored. Citrus greening disease is transmitted from infected citrus plants to healthy ones by colonization of infected *D. citri*. Therefore, agents that kill the psyllid at the flightless nymphal stages will effectively suppress spread of the disease.

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