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MORPHOLOGICAL AND MOLECULAR IDENTIFICATION OF *PARACOCCUS MARGINATUS* (HEMIPTERA: PSEUDOCOCCIDAE) IN YUNNAN, CHINA

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ABSTRACT

The papaya mealybug, *Paracoccus marginatus* Williams & Granara de Willink (Hemiptera: Pseudococcidae), highly polyphagous and now widespread, was discovered in Mengla County, Yunnan Province, China. The present study is aimed at confirming the identity of *P. marginatus* in mainland China based on its morphology and using molecular methods. Mitochondrial genes for cytochrome oxidase I (CO-I) were sequenced from 2 samples of *P. marginatus*. Homologous DNA sequences of *P. marginatus*, *Phenacoccus solenopsis* Tinsley and *Coccus viridis* (Green) (Hemiptera: Coccidae) - as an outgroup -were downloaded from Gen Bank. The morphology of putative *P. marginatus* specimens was examined using the same specimens as were subsequently used for the molecular study. Key morphological traits are described and illustrated. A 746 bp fragment of the CO-I was analyzed for the identification of *P. marginatus.* A phylogenetic tree was generated with the DNA sequences of all the mealybug species used in this study. The tree included 2 distinct clades: one consisting of the samples of *P. marginatus* and the other consisting of *P. solenopsis*. The phylogenetic tree and the sequences indicated that *P. marginatus* collected on papaya had 99% similarity with the other 3 accessions found through the BLAST search. Identification information may help strengthen quarantine programs to protect agroforestry production and maintain the ecological balance in China.

Key Words: papaya mealybug, morphological characterization, molecular identification, China

RESUMEN

La cochinilla de la papaya, *Paracoccus marginatus* Williams y Granara de Willink (Hemiptera: Pseudococcidae), altamente polífaga y ahora generalizada, fue descubierta en el Condado de Mengla, Provincia de Yunnan, China. El objetivo del estudio presente es confirmar la identidad de *P. marginatus* en la China continental en base a su morfología y el uso de métodos moleculares. Los genes mitocondriales para citocromo oxidasa I (CO-I) fueron secuenciados de 2 muestras de *P. marginatus.* Las secuencias de ADN homólogas de *P. marginatus*, *Phenacoccus solenopsis* Tinsley y *Coccus viridis* (Green) (Hemiptera: Coccidae) - como un grupo externo - fueron descargados de Gen Bank. Se examinó la morfología de los especimenes de las *P. marginatus* putativas utilizando las mismas muestras que se utilizaron posteriormente para el estudio molecular. Se describen e ilustran las caracteristicas morfológicas claves. Se analizó un fragmento de 746 pb de la CO-I para la identificación de *P. marginatus*. Un árbol filogenético fue generado con las secuencias de ADN de todas las especies de cochinillas harinosas utilizadas en este estudio. El árbol incluyó 2 clados diferentes: uno que consiste en las muestras de *P. marginatus* y la otra que consiste en *P. solenopsis*. El árbol filogenético y las secuencias indicaron que *P. marginatus* recolectadas sobre papaya tiene un 99% similitud con las otras 3 accesiones que se encuentran a través de la búsqueda de BLAST. Esta información sobre la identificación de estas especies puede ayudar a fortalecer los programas de cuarentena para proteger la producción agroforestal y mantener el equilibrio ecológico en China.

Palabras Clave: cochinillas papaya, caracterización morfológica, identificación molecular, China

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The papaya mealybug, *Paracoccus marginatus* Williams & Granara de Willink (Hemiptera: Pseudococcidae) is a small polyphagous sucking insect, which attacks various host plants including economically important tropical and subtropical fruits, vegetables and ornamental plants (Miller & Miller 2002). It damages the plants by sucking sap and injecting a toxic substance into the leaves. Furthermore, the its honeydew provides a substrate for the development of sooty mold fungi and decreases the quality of fruits and ornamental plants (Williams 1985; Franco et al. 2000). The infested leaves become crinkled, yellowish and withered. The honeydew produced by the mealybug and the associated black sooty mold impair the photosynthetic efficiency of the attacked plants.

The papaya mealybug is believed to be a native of Mexico and/or Central America. The pest were first collected in 1955 in Mexico, but it was described in 1992 from the neotropical regions in Belize, Costa Rica, Guatemala, and Mexico (Williams & Granara de Willink 1992). Papaya mealybug was recognized as a pest when it invaded the Caribbean region. It had been recorded in 14 Caribbean countries (Walker et al. 2006) by 1994. The pest was also recorded in Bradenton, Florida, in 1998 on *Hibiscus* (Miller et al. 1999), and by Jan 2002 it had spread to 18 plant species in 30 cities in the USA. The establishment of this mealybug in Guam in 2002 (Meyerdirk et al. 2004; Walker et al. 2006) and Palau in 2003 resulted in further spread to the Hawaiian Islands in the Pacific (Muniappan et al. 2006; Walker et al. 2006). In 2004, *P. marginatus* was recorded on papaya, plumeria, hibiscus, and *Jatropha* spp. L. in Hawaii (Heu et al. 2007). An outbreak was found by a team of scientists in the Integrated Pest Management Collaborative Research Support Program (IPM CRSP) at the Bogor Botanical Gardens in Java, Indonesia. Subsequent surveys revealed that it had spread to Bali and Sulawesi Islands. In July 2008, IPM CRSP scientists visiting Coimbatore, India found *P. marginatus* infesting papaya in an orchard at Tamil Nadu Agricultural University. It has since spread to 3 adjacent districts. In Sep 2008, IPM CRSP scientists also helped to identify *P. marginatus* in Sri Lanka, and in Nov 2008, *P. marginatus* was reported in northern Thailand. In May 2009, IPM CRSP scientists found *P. marginatus* at Joydebpur, Bangladesh, and in Aug 2009, it was confirmed in the Maldives (Muniappan et al. 2008; Mani et al. 2013), and in 2009 was reported from Malaysia. In Sri Lanka, papaya mealybug has been spread mainly to Gampaha, Colombo, Kurunagela and Kalutera districts. (Galanihe et al. 2010). Although papaya mealybug has therefore spread to many countries in Asia, it has not been previously reported from China.

A survey was therefore undertaken to determine whether the papaya mealybug was present in Yunan Province (N 22.48401° E 101.56245°) , China, and it was located on papaya (*Carica papaya* L.; Brassicales: Caricaceae) plantations and on *Plumeria* spp. (Gentianales: Apocynaceae) in Oct 2012. The mealybugs were found scattered on all above-ground parts of the plant, on which it caused serious damage. Samples were collected, preserved in ethyl alcohol (95%) and stored at 20 °C until further use. In this study, we used morphological characterization and molecular methods to identify *P. marginatus* in Mainland China.

Materials and Methods

Morphological Examination

We morphologically examined at least one specimen from each voucher sample. The mealybugs were prepared for slide-mounting and determined to be the papaya mealybug, *P. marginatus* by Professor San-an Wu, Beijing Forestry University. The slide-mounting process was as follows: (a) after making a small incision on the back of papaya mealybug, it was heated in 10% NaOH for 10 min; (b) the body contents was expelled and the body was gently flattened with a microspatula; (c) the preparation was stained morphologically in a saturated solution of fuchsine in a mixture of distilled water, lactic acid and glycerol (1:1:1) for 3-5 h; (d) washed in glacial acetic acid for 1 h to stabilize the staining, transferred to lavender oil, allowed to stand for at least 1 h, and (f) placed in a drop of Canada balsam on a slide and covered with a cover glass. The slide was then labeled and examined by a compound microscope. Identification was based on the taxonomic keys of Williams & Granara de Willink (1992) and Miller & Miller (2002).

DNA Extraction and PCR Amplification

Samples were rinsed with double distilled water and dried prior to DNA extraction. All samples were examined under the microscope to check for the presence of parasitoids. The total genomic DNA was extracted from the whole body in 95% ethanol with DNeasy Blood &Tissue Kit (Qiagen, Alameda, California) according to the manufacturer's instructions. Standard protocols were adopted in DNA extraction, polymerase chain reaction, sequencing the mitochondrial cytochrome oxidase I (CO-I) gene fragment and sequence alignment (Hajibabaei et al. 2005). DNA concentration and purity were estimated by means of absorbance at 260 nm to absorbance at 280 nm in a NanoDrop ND-2000 (NanoDrop Technologies, Wilmington, Delaware, USA) spectrophotometer. The primers C1J2195 TTGATTYTTTGGTCATCCAGAAGT and TL2N3014 TCCAATGCACTAATCTGCCATATTA (Chris et al. 1994) were initially used to amplify 746bp of the mitochondrial

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CO-I gene. All samples were successfully amplified. The reaction was conducted in a thermal cycler in a 30 µL system composed of 3 µL of PCR buffer, 2.4 µL of dNTP mixture, 0.15 µL of Taq DNA polymerase, 1 µL of each primer, 1.8 µL of the DNA template, and ddH_2O added to a final reaction volume of 30 µL. The PCRs were conducted in Eppendorf Mastercycler Thermal Cyclers by using the following procedure: an initial step of 4 min at 94 °C, followed by 35 cycles of 30 s at 95 °C, 45 s at 48 °C, and 1 min at 72 °C, which was followed by a final 5-min extension at 72 °C. The PCR products were evaluated by loading 3 µL of the product on 1.2% agarose gel. DNA identity was then confirmed via sequencing by using an ABI 3730xl sequencer (BGI Life Tech Co. Ltd., Shenzhen, China).

The sequences of the mealybug species were deposited in Gen Bank under accession Nos. KJ187495 and KJ187496 (CO-I), and the other CO-I sequences of *P. marginatus, Phenacoccus solenopsis* and *Coccus viridis* (*Coccus viridis* used as an outgroup) were obtained from GenBank, with accession Nos. JF933768, KF686748, EU267201, KF878038, KF878039, KF878042, KF878059 and GU936953. Sequences were generated in the present study along with those of *P. solenopsis* and *C. viridis.* The voucher samples were stored in the Plant Quarantine Laboratory of Guangdong Entry-Exit Inspection and Quarantine Bureau (GDCIQ), China.

Data Analysis

We performed a Blast search on NCBI (http://www.ncbi.nlm.nih.gov/) after obtaining the sequence of the PCR products. Once the high identity score (above 99%) of the *P. marginatus* CO-I gene was attained, we aligned the sequence data by ClustalW2 (Thompson et al. 1994) at the default parameter settings. Small changes were made to the alignment. The hyper-variable regions were excluded from further analysis because of the ambiguity of the alignment (Swofford et al. 1996). Phylogenetic analysis of aligned sequences was done using MEGA. 5.0 (Tamura et al. 2011). The method of neighbor-joining (NJ) with the Kimura two- parameter model (Kimura 1980) was selected to build the phylogenetic tree. To assess the robustness of the phylogenetic tree, 1000 bootstrap replicates were selected.

RESULTS

Morphological Characters

The morphological characters that might be useful for identifying adult female *P. marginatus* are as follows: body elongate oval, 8 antennal segments, 17 pairs of cerarii, oral-rim tubular ducts restricted to margin and sub-margin on dorsum, presence of translucent pores only on hind coxa, these unusually large and abundant, ventral oral-rim tubular ducts present on lateral areas of the thorax, ventral multilocular pores absent from lateral areas of abdomen, anal bar present ventrally on each anal lobe, and dorsal setae generally equal to or shorter than conical cerarian setae (Fig. 1).

This invasive mealybug has a soft body that is pale yellow in color, about 2-3mm long and 1.5mm wide, and covered with mealy white wax. The surface wax on the dorsum showed transverse creases between the body segments. The last instar of

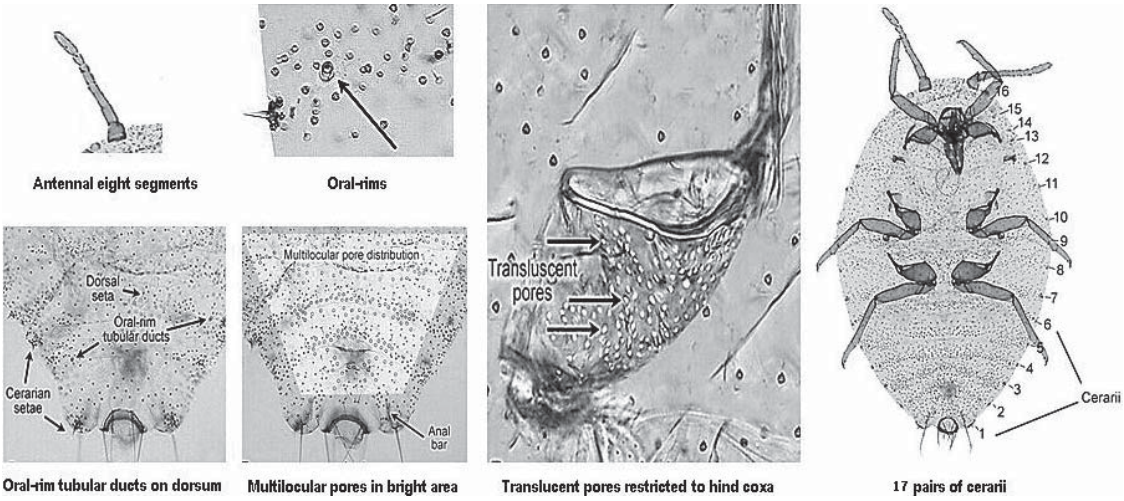

Fig 1. Morphological characters for the identification of *Paracoccus marginatus* Williams & Granara de Willink, collected on *Carica papaya* and *Plumeria* spp. from Mengla County, Yunan Province, China.

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the female secreted an ovisac of white wax filaments from the ventral margins of the abdomen, which eventually extended 3 to 4 times the body length and entirely covered the female.

Molecular Identification

Mitochondrial cytochrome oxidase I (CO-I) was successfully sequenced from an individual *P. marginatus*. A comparison of the triplicate sequences showed no evidence of mismatch indicating sequence error. A 746 bp fragment of the CO-I was analyzed for the identification of *P. marginatus.* The sequences of *P. marginatus* have 5 variable sites at the 41, 49, 713,740 and 742 sites. *P. marginatus* showed 12.7% (95/746) sequence variation with *P. solenopsis*. *Coccus viridis* was selected as an outgroup.

We generated the NJ trees by using the aligned CO-I sequence. The phylogenetic tree generated with the sequences of all the mealybugs used in the present study is shown in Fig. 2. The phylogenetic tree and the sequences indicated that *P. marginatus* collected on papaya had 99% similarity with the other 3 accessions found through the BLAST search and used for constructing the phylogenetic tree. The tree included 2 distinct clades: one clade consisting of the samples of *P. marginatus* and the other clade consisting of *P. solenopsis*.

Discussion

Paracoccus marginatus, infested the veins of older leaves and all parts of young leaves and fruits. Honeydew excreted by this mealybug results in the development of sooty mold that covers the leaves, fruit and stems, impeding photosynthesis and gaseous exchange. Serious damage was observed on the papaya plants caused by this new mealybug, so it is important to identify it. From the examination of slide-mounted specimens, morphological characters were found to be similar to the descriptions of the adult female papaya mealybug by Miller & Miller (2002) and Williams & Granara de Willink (1992). Live adult female mealybugs have no wings and are characterized by a yellow body, light yellow legs, and a mealy wax covering on the body, which is not thick enough to hide the yellow body. No bare areas were observed on the dorsum. Only a ventral ovisac was observed. Live adult female mealybugs have 15 to 17 lateral wax filaments, the posterior pair is conspicuously longer than the others being about 1/8 of the length of the body. Samples of papaya mealybug turn black when immersed in 95% alcohol.

The papaya mealybug is sometimes associated with other mealybugs, such as the pink mealybug, *Maconellicoccus hirsutus* (Green). The following 2 characteristics are important for distinguishing *P. marginatus* adult females from all other species of *Paracoccus*. Firstly, the oral-rim tubular ducts are dorsally restricted to the marginal areas of the body and the translucent pores are absent from the hind tibiae. Secondly, adult males may be distinguished from other related species by the presence of stout fleshy setae on the antennae and the absence of fleshy setae on the legs (Miller & Miller 2002).

Scale insects are notorious as invasive species because of their small size and their affinity for settling in cryptic areas of the host. Molecular methods have been successfully used to identify immature as well as adult mealybugs and have been used to clarify the invasion history of exotic species. Molecular identification is particularly useful, because it is not limited to only or two life stages but can be applied to the egg, larva, pupa and adult, without regard to polymorphism and gender (male or female) of the insect.

Paracoccus marginatus is one of the most widespread mealybugs, and it causes significant dam-

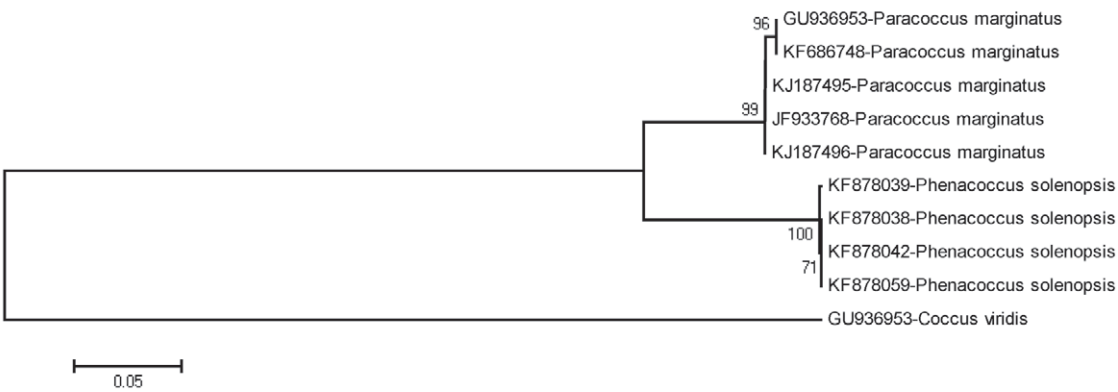

Fig 2. Neighbor joining (NJ) tree (MEGA 5.0) with bootstrap support (1000 replicates) indicating the clustering situation of mealybugs for CO-I sequences. All the sequences of *Paracoccus marginatus* formed a single clade with more than 99% similarity in sequences. *Coccus viridis* (GU936953) was used as an outgroup.

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age to fruits, vegetables and ornamental plants in many countries throughout the subtropics and tropics. The papaya mealybug is polyphagous and has been recorded on over 60 species of plants in more than 25 genera (Meyerdirk & Kauffman 2001). This paper reports the papaya mealybug in Mainland China for the first time. The source of this species in China is not clear at present. In the future, we will collect more papaya mealybug samples in tropical and subtropical region of China, i.e., Yunnan Province, Guangxi Province, Guangdong Province, Hainan Province, and Fujian Province, and we will analyze the genetic differentiation and genetic structure of populations in China.

Our studies used morphological characterization and molecular methods to identify papaya mealybugs, *P. marginatus* in mainland China. The molecular results were confirmed by the examination of slide-mounted samples. Identification information of this species will help us strengthen quarantine programs so that inspectors and identifiers will be able to determine the species and make informed control decisions at Chinese ports.

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