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Authors: He, Yan Biao, Wan, Xuan Wu, Liu, Yin Hong, Sun, Guang Ming, and Zhan, Ru Lin

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MITOCHONDRIAL COI FROM *DYSMICOCCLUS BREVIPES* (HEMIPTERA: PSEUDOCOCCIDAE) SUGGESTS CRYPTIC LINEAGE AND PINPOINTS THE SOURCE OF THE INTRODUCTION TO CHINA

YAN BIAO HE^{1,2,*}, XUAN WU WAN¹, YIN HONG LIU^{1,*}, GUANG MING SUN², AND RU LIN ZHAN²

¹Chongqing Key Laboratory of Entomology and Insect Control Engineering, College of Plant Protection, Southwest University, Chongqing, the People's Republic of China

²South Subtropical Crops Research Institute, Chinese Academy of Tropical Agricultural Sciences, Zhanjiang, the People's Republic of China

*Corresponding author; E-mail: heyانبiao@yahoo.com.cn, yhliu@swu.edu.cn

ABSTRACT

The pink pineapple mealybug, *Dysmicoccus brevipes* (Cockerell) (Hemiptera: Pseudococcidae), is a widespread plant-sucking insect of considerable concern because it transmits the pineapple mealybug wilt-associated virus. The purpose of this study was to use molecular methods to clarify the relationship among pink pineapple mealybugs from China, Philippines, Thailand and Hawaii, in an effort to determine the geographic origin of the introduction to China. Pink pineapple mealybug samples were collected from 10 counties of 5 provinces in China, and samples from Hawaii were obtained. Parts of the mitochondrial genes for cytochrome oxidase I (COI) were sequenced for each sample, homologous DNA sequences of samples from the Philippines and Thailand were downloaded from GenBank. Three haplotypes were found in China, 1 from populations of mainland China, and 2 from Hainan island. Phylogenetic analyses suggested that most pink pineapple mealybugs on mainland China probably were closely related to populations in the Republic of the Philippines. However mealybugs from Wanning, Hainan, China, represented a different lineage that clearly diverged from other populations, and which may be of a cryptic lineage or species in the pink pineapple mealybug complex. The probable geographic origin of pink pineapple mealybugs in China is discussed.

Key Words: pink pineapple mealybug, molecular phylogenetics, cryptic lineage

RESUMEN

La cochinilla rosada de la piña, *Dysmicoccus brevipes* (Cockerell) (Hemiptera: Pseudococcidae), es un insecto chupador de plantas de amplia distribución que genera una considerable preocupación porque transmite el virus de la marchitez de la piña. El propósito de este trabajo fue usar técnicas moleculares para estudiar la relación entre cochinillas rosadas de China, Filipinas, Tailandia y Hawaii, y así determinar el origen geográfico de cochinillas introducidas a China. Se obtuvieron cochinillas de 10 condados de 5 provincias de China, y muestras de Hawaii. Partes de los genes mitocondriales de citocromo oxidasa I (COI) fueron secuenciados para cada muestra y secuencias homólogas de Tailandia y Filipinas fueron obtenidas del GeneBank. Se encontraron tres haplotipos en China, uno de la parte continental de China, y dos de la isla de Hainan. Los análisis filogenéticos sugieren que la mayoría de las cochinillas rosadas de la piña en la parte continental de China se relacionaban estrechamente con la población de la República de las Filipinas. Sin embargo, las cochinillas de Wanning, en la Provincia de Hainan, China, representan un linaje diferente que claramente divergió de otras poblaciones, el cual puede ser un linaje o taxón críptica dentro del complejo de cochinillas rosadas de la piña. Se discute el posible origen geográfico de las cochinillas rosadas de piña en China.

Ito (1938) found that 2 strains of mealybug commonly infesting pineapple *Ananas comosus* (L.) Merr. (Poales: Bromeliaceae) in Hawaii; he believed that both were strains of the pineapple mealybug, *Dysmicoccus brevipes* (Cockerell) (Hemiptera: Pseudococcidae). Several decades later Beardsley (1959; 1965) concluded that these 2 strains were 2 different species, i.e., *D. brevipes*, the parthenogenetic pink pineapple mealybug,

and *Dysmicoccus neobrevipes* Beardsley, the bisexual gray pineapple mealybug. During the past century, these 2 species have been of great concern, mainly because they have been implicated in transmitting pineapple mealybug wilt-associated virus (Rohrbach et al. 1998; Sether et al. 1998; Sether 2002). Closterovirus infection and mealybug exposure were found to be necessary for the development of mealybug wilt disease of

pineapple, also known as mealybug wilt in Hawaii (Sether & Hu 2002; Sether et al. 2005). In last 5 yr, *D. neobrevipes* became widely known in south China for being seriously damaging to agave, *Agave sisalana* Perrine (Asparagales: Asparagaceae). Pineapple mealybugs are commonly found on pineapple in southern China, but it is not known if there are only 1 or 2 species of mealybug, i.e., *D. brevipes* and *D. neobrevipes*.

Molecular methods have been used very successfully in helping to determine the invasion history of invasive taxa (Downie 2002; Saltonstall 2002; Facon et al. 2003; Cognato et al. 2005). *Dysmicoccus brevipes* is one of the most widespread mealybugs, throughout the tropic and temperature areas (Williams and Watson 1988; Sether 2002), and is believed to be native to South America. However the relationships among pink pineapple mealybugs worldwide have not been elaborated. In this study, we used molecular methods to clarify the relationships among pink pineapple mealybug populations in China, Republic of the Philippines, Kingdom of Thailand and Hawaii, and to analyze the geographic origins of the populations that have invaded China. We sampled 14 mealybug populations from 5 provinces in China (Fig. 1), and 1 population from Hawaii. A 948 bp region of mitochondrial DNA (mtDNA) was sequenced for each sample. Homologous DNA sequences of samples from the Philippines and

Thailand were downloaded from Genbank and analyzed as part of this study.

MATERIALS AND METHODS

Collection and Handling of Mealybugs

Pineapple mealybugs on pineapple were obtained from 12 counties in 5 provinces, and *D. brevipes* was obtained from Hawaii. In addition *D. neobrevipes* and *Planococcus minor* (Maskell) from China were included as outgroups. Samples were collected during 2010 and 2011 (Table 1). For species identification, 18S and 28S sequence alignments were done at the time of morphological examination by Professor Sanan Wu, Beijing Forestry University, and an expert in the taxonomy of Coccoidea. The primers of 18S and 28S were from Downie et al. (2004) and Dietrich et al. (2001) respectively.

Live insects were carefully removed from the host plant and held without a food for 24 h before being stored in 95% ethanol at 4 °C. Voucher samples have been preserved at the South Subtropical Crops Research Institute, Chinese Academy of Tropical Agricultural Sciences.

Samples were rinsed with double distilled water and dried before DNA extraction. Prior to DNA



Fig. 1. Map showing the distribution of *Dysmicoccus brevipes* in China.

TABLE 1. COLLECTION INFORMATION FOR SAMPLES OF *DYSMICOCOCCUS BREVIPES* AND OUTGROUP SPECIES.

Population name	Sample name	Host Species	Collection information
<i>Dysmicoccus brevipes</i>			
Jinghong	YN1	<i>Ananas comosus</i>	CHINA; Yunnan Province, Jinghong County, Ganlanba; 27 Jul 2010; Coll. Y. B. He
Jinggu	YN2	<i>Ananas comosus</i>	CHINA; Yunnan Province, Jinggu County, Jinggu; 29 Jul 2010; Coll. Y. B. He
Longzhou	GX1	<i>Ananas comosus</i>	CHINA; Guangxi Province, Longzhou County, Xianghui; 26 June 2010; Coll. Y. B. He
Nanning	GX2	<i>Ananas comosus</i>	CHINA; Guangxi Province, Nanning County, Longan; 25 June 2010; Coll. Y. B. He
Pubei	GX3	<i>Ananas comosus</i>	CHINA; Guangxi Province, Pubei County, Pubei; 27 June 2010; Coll. Y. B. He
Xuwen	QJ1	<i>Ananas comosus</i>	CHINA; Guangdong Province, Xuwen County, Qujie; 17 Oct. 2010; Coll. Y. B. He
Leizhou	GD1	<i>Ananas comosus</i>	CHINA; Guangdong Province, Leizhou County, Tiaofen; 17 Oct. 2010; Coll. Y. B. He
	GD2	<i>Ananas comosus</i>	CHINA; Guangdong Province, Leizhou County, Tiaofen; 17 Oct. 2010; Coll. Y. B. He
	GD3	<i>Ananas comosus</i>	CHINA; Guangdong Province, Leizhou County, Tiaofen; 17 Oct. 2010; Coll. Y. B. He
Wanning	WN1	<i>Ananas comosus</i>	CHINA; Hainan Province, Wanning County, Wanning; 21 Aug. 2010; Coll. Y. B. He
Qionghai	QH1	<i>Ananas comosus</i>	CHINA; Hainan Province, Qionghai County, Qionghai; 21 Aug. 2010; Coll. Y. B. He
	HN1	<i>Ananas comosus</i>	CHINA; Hainan Province, Qionghai County, Qionghai; 21 Aug. 2010; Coll. Y. B. He
Zhangzhou	FJ1	<i>Ananas comosus</i>	CHINA; Fujian Province, Longhai County, Chengxi; 18 July 2010; Coll. Y. B. He
	FJ2	<i>Ananas comosus</i>	CHINA; Fujian Province, Longhai County, Chengxi; 18 July 2010; Coll. Y. B. He
Hawaii	HI1	<i>Ananas comosus</i>	USA; Hawaii, Nov. 2010; Coll. D. M. Sether
<i>Dysmicoccus neobrevipes</i>			
<i>D. neobrevipes</i>	<i>D. neobrevipes</i>	<i>Agave sisalana</i>	CHINA; Guangdong Province, Leizhou County, 30 Sept. 2010; Coll. Y. B. He
<i>Planococcus minor</i>			
<i>P. minor</i>	<i>P. minor</i>	<i>Mangifera indica</i>	CHINA; Guangdong Province, Xuwen County, 12 June 2010; Coll. Y. B. He

extraction, all specimens were examined under the microscope for the presence of parasitoids. Total DNA was extracted from single parasitoid-free adult females using the TIANamp Genomic DNA kit (Tiangen Biotech Co., Ltd., Beijing, China).

Template Preparation and DNA Manipulation

Six hundred and forty nine bp of the mitochondrial cytochrome c oxidase subunit I (COI) gene were amplified with the primers (F: 5'-CCTTCAACTAATCATAAAAAATATTAG -3') and (R: 5'- TAAACTTCTGGATGTCCAAAAAATCA 3') (Park et al. 2010). PCR assays were performed in 25 µL reaction volumes with 3 µL of template DNA. The PCR reaction mix contained 2.5 µL 10 × PCR Taq reaction buffer (Promega, Madison, Wisconsin), 0.5 U Taq DNA polymerase (Promega), 3.5 mM MgCl₂, and 15 pmol of each primer. The PCR reaction program was initialized at 95 °C for 3 min, followed by 34 cycles at 95 °C for 30 s, 52 °C for 45 s, 72 °C for 1 min, and a final extension of 5 min at 72 °C.

Five microliters of each PCR product were run on a 1% agarose gel to determine the presence and size of amplified DNA. PCR products were sequenced in both forward and reverse directions. The sequencing of PCR products was carried out on an ABI 377 automated sequencer. Sequences of all haplotypes of mealybug species have been deposited in GenBank under accession Nos. JN128957-JN128963 (COI) (Table 2). The COI sequences of *D. brevipipes* in Thailand and the Philippines were obtained from GenBank with accession Nos. HM474137, HM474139 and HM474142-HM474145.

Data Analysis

Sequences were aligned using ClustalX (ver. 2.0) and unique haplotypes were identified with ARLEQUIN (ver. 3.5). Descriptive statistics (number of variable sites, number of haplotypes, haloptype diversity, nucleotide diversity, average number of nucleotide differences between haplotypes) were calculated using DNASP (ver. 5.0).

A network can be a more appropriate way of depicting intraspecific gene genealogies than a bifurcating tree because of the potential for extinct ancestral nodes and multifurcating relationships (Posada & Crandall, 2001). We therefore examined the genealogical relationships among pink pineapple mealybugs mtDNA haplotypes by constructing a network based on the statistical parsimony method of Templeton et al. (1992), using the software TCS 1.20 (Clement et al., 2000).

Phylogenetic analyses were performed by maximum parsimony (MP) and neighbor joining (NJ) by using PAUP* 4.10 (Swofford 2003).

TABLE 2. VARIABLE SITES OF COI GENE SEQUENCES IN VARIOUS POPULATIONS OF *DYSMICOCCLUS BREVIPES*.

Haplo-type	Samples included	34	49	103	139	277	283	311	325	347	367	476	478	480	482	506	589	601
QJ	YN1,YN2,GX1,GX2,GX3,QJ1,GD1,FJ1	T	T	C	A	A	G	A	T	T	C	T	A	G	A	A	C	T
QH	QH1,GD2,FJ2	•	•	T	•	•	•	G	•	•	•	•	A	A	A	G	•	•
WN	WN1,HN1,GD3	A	A	T	T	C	A	G	•	C	T	A	T	A	G	•	T	C
HI	HI1, TH*	•	•	•	•	•	•	G	•	•	•	•	•	A	•	•	•	•
PH	PH*	•	•	•	•	•	•	G	•	•	•	•	•	A	•	•	•	•

Shared haplotypes include samples with identical sequences across all mitochondrial regions included in this study, and are named after the abbreviation of the representative region on the list. Haplotypes that occurred only once were given the same name as the sample in which they were found (Table 1). QJ: Qujie, Xuwen, Guangdong, China; QH: Qionghai, Hainan, China; WN: Wannan, Hainan, China; HI: Hawaii, USA; TH: Thailand; and PH: the Philippines (same as below). *represents COI sequences obtained from GenBank, and “•” means the base at this site was identical to that in the QJ haplotype.

Incongruence among data partitions was tested using a partition homogeneity test with 1,000 replications as implemented in PAUP*. MP and NJ analyses used the heuristic search option with tree-bisection-reconnection (TBR), branch swapping, collapsing zero-length branches, and equal weighting of all characters. MP bootstrap support was calculated using 1,000 repetitions.

RESULTS

Both 18S and 28S sequences and morphological examination were applied to differentiate between the 2 species of pineapple-infesting mealybugs, *D. brevipes* and *D. neobrevipes*. A pineapple mealybug complex was not found. Ten of 11 populations of pineapple mealybugs from mainland China were identified as *D. brevipes*, and the 11th one was identified as *D. neobrevipes*. The Accession Nos. in GenBank of 18S sequences of the Wanning population and other populations of *D. brevipes* were JF965398, JF965399 respectively, and that of *D. neobrevipes* is JF965400. The Accession Nos. in GenBank of the 28S sequences of *D. brevipes* and *D. neobrevipes* were JF965409 and JF965410, respectively.

The *D. brevipes* samples from mainland China were found to include 3 COI haplotypes, characterized by 17 variable sites of COI (Table 2). Two other haplotypes were observed among the samples from Hawaii, Thailand and Philippines; with one of them being found in both Hawaii and Thailand. Most pink pineapple mealybug populations in mainland China possessed haplotype QJ, while most samples possessing haplotypes QH and WN were from the Hainan Island (Table 2).

In an attempt to determine the source of the introduction of *Dysmicoccus* to China, the COI

sequences we analyzed included not only all of the *D. brevipes* samples we had obtained, but also those from Thailand and the Philippines. The two were obtained by downloading homologous mDNA sequences from Genbank.

Haplotype QJ (QuJie, Xuwen, Guangdong, China) was the predominant haplotype in mainland China, with a frequency of 0.635; while haplotypes QH (Qionghai, Hainan, China) and WN (Wanning, Hainan, China) had the frequencies of 0.148 and 0.130, respectively (Table 3). Haloptype diversity and nucleotide diversity were 0.743 and 0.00934, respectively; and the average number of nucleotide differences was 5.779 (Table 3).

The genetic distances between different haplotypes of *D. brevipes* varied from 0.001 to 0.015 (Table 4), of which that between haplotype WN and haplotype QJ was the greatest. The *D. brevipes* population of Hawaii was closely related to the population of the Philippines (Fig. 2). Haplotype QJ, found in most mealybug samples in mainland China, had the same genetic distance as haplotype QH to haplotype PH found in the population in the Philippines. The haplotype network revealed a very high divergence between the haplotype WN and all other haplotypes (Fig. 2).

Pink pineapple mealybugs clustered into 4 major clades based on the sequences of COI. The first clade, QJ (Fig. 3) includes most samples collected in mainland China. The second clade (QH) includes samples collected in Qionghai, Hainan Island, and in some of the samples collected in Guangdong and Fujian provinces. The third, WN clade, consisted of samples collected in Wanning, Hainan Island, and some samples collected in Guangdong province. The fourth, HI clade, consisted of samples collected in Hawaii. As seen

TABLE 3. DISTRIBUTION FREQUENCIES OF DIFFERENT HAPLOTYPES IN GEOGRAPHICAL POPULATIONS OF *DYSMICOCOCCUS BREVIPEDES*.

Population name	Number of samples per population	Haplotypes			
		QJ	QH	WN	HI
Jinghong	20	20			
Jinggu	20	20			
Longzhou	20	20			
Nanning	20	20			
Pubei	20	20			
Xuwen	20	20			
Leizhou	26	10	12	4	
Wanning	20			20	
Qionghai	24		18	6	
Zhangzhou	20	16	4		
Hawaii	20				20
Frequency of distribution		0.635	0.148	0.130	0.087
Haloptype diversity		0.743			
Nucleotide diversity		0.00934			
Average number of nucleotide differences		5.779			

Abbreviations: QJ: Qujie, Xuwen, Guangdong, China; QH: Qionghai, Hainan, China; WN: Wanning, Hainan, China; HI: Hawaii, USA.

TABLE 4. GENETIC DISTANCES BETWEEN DIFFERENT HAPLOTYPES OF *DYSMICOCOCUS BREVIPEDES*.

	QH	QJ	HI	PH	WN
QH	—				
QJ	0.004	—			
HI	0.003	0.003	—		
PH	0.002	0.002	0.001	—	
WN	0.013	0.015	0.014	0.013	—

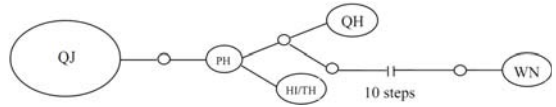


Fig. 2. TCS haplotype network showing genealogical relationships among various populations of *Dysmicoccus brevipes*. Haplotypes are connected with a 90% CL. White dots represent mutational steps separating the observed haplotypes, and the size of each oval is proportional to the frequency of the haplotype in the analysis.

in Fig. 2, the QJ (QuJie, Guangdong,) clade and the QH (Qionghai, Hainan) clade had a close genetic relationship with the Hawaii clade, and WN (Wanning, Hainan) clade is distantly related to the Hawaii clade (Figs. 3 and 4).

DISCUSSION

The relationships among mitochondrial lineages uncovered for pink pineapple mealybug

populations on mainland China revealed previously unknown biogeographic patterns. Three haplotypes were found in China, 1 from mainland China, and 2 from the island of Hainan. Pink pineapple mealybugs collected in Yunnan province, Guangxi province and most samples in Guangdong and Fujian provinces all shared an

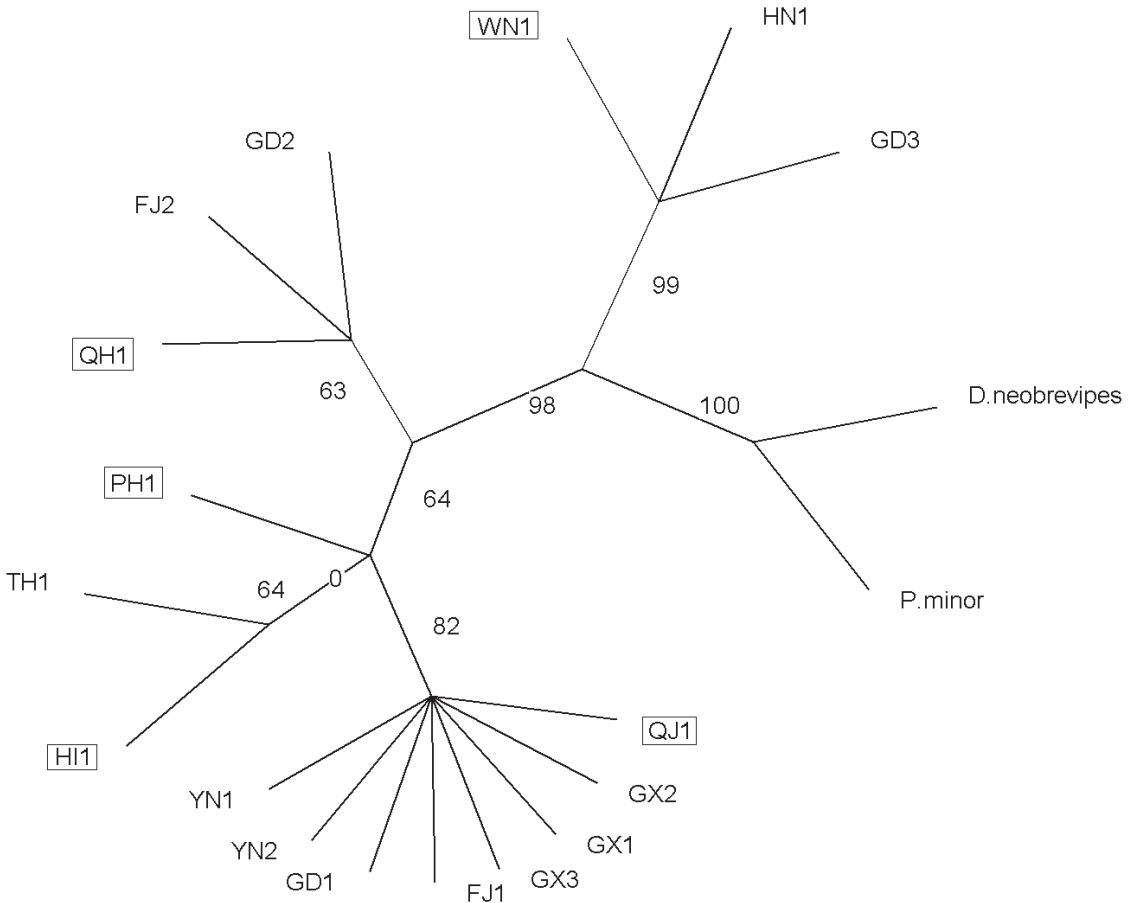


Fig. 3. The maximally parsimonious tree of 15 population of *Dysmicoccus brevipes*. PAUP 4.10 software was used for the analysis. The 2 species, *Dysmicoccus neobrevipes* and *Planococcus minor* were included as outgroups based on the combined COI sequences.

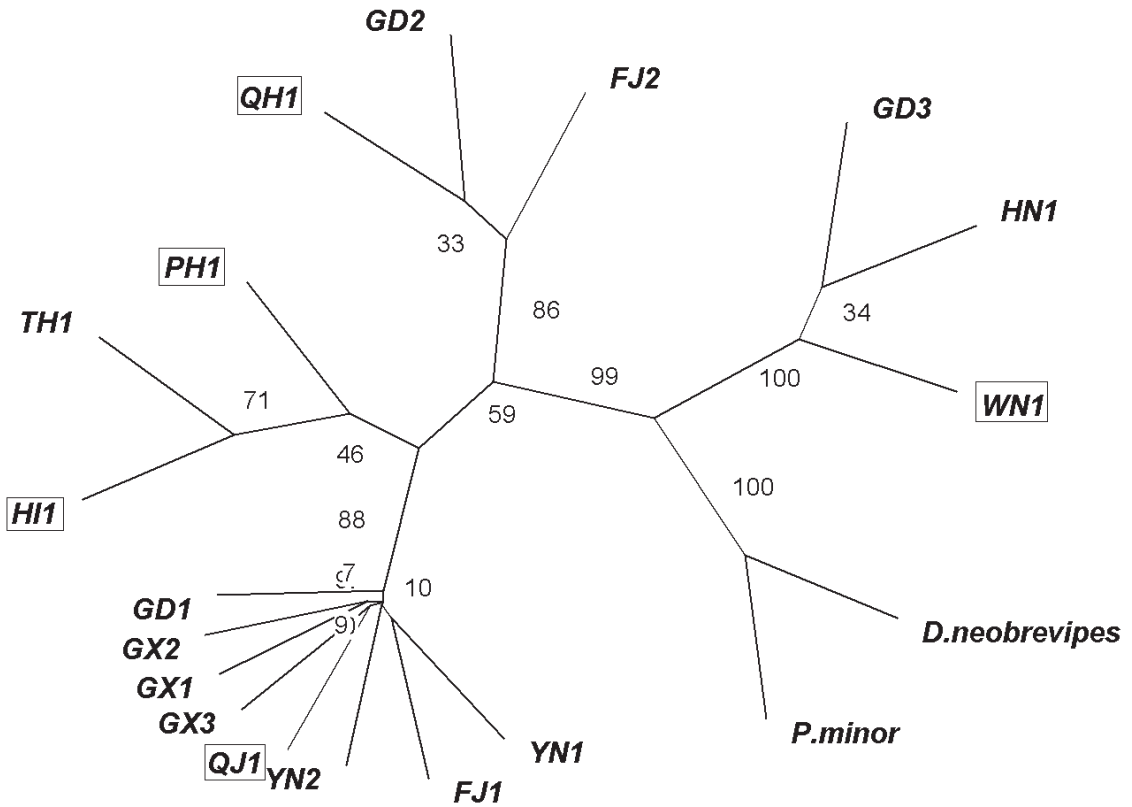


Fig. 4. The neighbor joining tree of 15 populations of *Dysmicoccus brevipes*. PAUP 4.10 software was used for the analysis. The two species, *Dysmicoccus neobrevipes* and *Planococcus minor* were included as outgroups based on COI sequences.

identical haplotype, suggesting limited genetic variation in mealybugs in mainland China.

However, pink pineapple mealybugs from Wanning differ greatly from other populations in China by 0.015 in genetic distance and 16 steps in TCS haplotype network (Fig. 2). But morphological examination did not confirm that the pink pineapple mealybug from Wanning is a different species. Also pink pineapple mealybugs from Wanning share the same fragment of 28S with other populations, and the fragment of 18S is only slightly different with one base transition at the 271st base position. The large genetic distance between the collections from Wanning and those from other populations suggest that *D. brevipes* might be a species complex, which includes at least two cryptic lineages or sibling species or other taxa. The pink pineapple mealybugs from Wanning are of a cryptic lineage, and may be a complex of taxa.

Phylogenetic analyses suggested the source of *D. brevipes* in Hawaii was very likely the mealybug lineage living predominantly in Thailand, and

that the mealybugs sharing an identical haplotype in mainland China were most closely related to the mealybug population in the Philippines, followed by Hawaii and then Thailand. These findings are consistent with the fact that overseas Chinese introduced a series of pineapple varieties to Guangdong province from countries in south Oceania and southeast Asia in the early 20th century. Thus, the pink pineapple mealybugs probably were introduced inadvertently on the pineapple seedlings. Possibly the mealybug population at Qionghai is also the result of an introduction in the early 20th century, because it has as close genetic relationship with Philippines population. In contrast to Qionghai, there is no conclusive evidence for a recent introduction of *D. brevipes* into Wanning. Possibly the lineage present in Wanning was introduced from an as yet unsampled native lineage in South America or Europe. Also, it seems reasonable to contend that the Wanning population had been established long before the introductions of the early 20th century.

The distribution map of different *D. brevipes* haplotypes (Fig. 1) probably conforms to the route

of initial dissemination of pineapple seedlings in China. Two of 3 *D. brevipipes* haplotypes in China were also found in Hainan Province, but the most widespread *D. brevipipes* haplotype in mainland China was not found on the island of Hainan, because Hainan has not introduced pineapple seedlings from mainland China. On the other hand many pineapple seedlings from Hainan are introduced to Leizhou, Guangdong province every year. Hence, it is likely that *D. brevipipes* populations displaying the 3 haplotypes (GD1, GD2, GD3) had mixed origins, notably with some introductions from Hainan Island. If pineapple seedlings continue to be disseminated throughout the mainland from the Leizhou Peninsula without stringent quarantine measures, the 3 haplotypes of Hainan may progressively invade the mainland.

The potential impact of such an expansion nonetheless depends on the possible differences in the biology of the populations displaying the various haplotypes. To characterize all *D. brevipipes* populations in China and worldwide, and how populations of different haplotype differ in their biology and taxonomy will require much additional research (Havill et al. 2006). Evidence in this study is insufficient to know if temperature or growth conditions optimal for the thriving and distribution of the different haplotypes of *D. brevipipes* are seriously affected by people. We detected 2 different haplotypes each a pineapple field at Qionghai and at Zhangzhou, and we detected 3 different haplotypes in one pineapple field at Leizhou. The mitochondrial DNA (COI or Cytb) sequences of mealybugs in these fields should be analyzed in the future in order to gather evidence for either parthenogenetic or bisexual reproduction in China, or both.

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