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Phylogeography of pink pineapple mealybugs, *Dysmicoccus brevipes* (Cockerell) reveals the history of pineapple introduction and cultivation in China

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ABSTRACT. The pink pineapple mealybug (PPM), *Dysmicoccus brevipes* (Cockerell) (Hemiptera: Pseudococcidae), is a widespread plant-sucking insect of considerable concern because it transmits the pineapple mealybug wilt-associated virus. Its distribution is closely linked with its host, the pineapple [*Ananas comosus* (L.) Merrill] because of its wingless and parthenogenetic characteristics. To investigate the history of *D. brevipes* introduction and the cultivation of pineapple in China, samples of *D. brevipes* were collected from the main pineapple production region in China, and from Thailand, and the mitochondrial cytochrome c oxidase subunit I (COI) gene was analyzed. Homologous sequences of *D. brevipes* COI from Brazil, Thailand, and Philippines that are deposited in GenBank were compared. Phylogenetic analyses suggest there are close genetic relationships between PPM populations from Hawaii, Brazil, the Philippines, and from Thailand and China, which probably originate from South America. It is suggested that

most PPMs in China were introduced from South America by way of Southeast Asia, being accompanied by the pineapple seedling. Conversely, some PPMs represented by Haplotype-WN from Wanning of China, and Lampang of Thailand were found to differ greatly from populations in Hawaii, Brazil, the Philippines, Thailand, and China. It is possible that another route was used for the introduction and distribution of pineapple, or that pineapple might have originated in Southeast Asia.

Key words: Pink pineapple mealybug; *Dysmicoccus brevipes* (Cockerell); Haplotype; Introduction and cultivation; Pineapple

INTRODUCTION

The pineapple [*Ananas comosus* (L.) Merrill] is a tropical plant of high economic importance worldwide, especially in terms of its large area of cultivation, high yield, and excellent consumer market (Lacerda et al., 2009), and it is believed to be of South America origin. Suckers or ratoons pulled from the base of plants are the most typical planting materials used for commercial production in Southeast Asia, for the sake of faster growth and easier field management. It is therefore very important to dip the planting material into a fungicide/insecticide mixture several days before planting to prevent the introduction of pests to a new field.

Pink pineapple mealybug [PPM; *Dysmicoccus brevipes* (Cockerell)] is a major pest of pineapples in many countries, and is normally found on the base of the leaf and stem or on the roots of the plant. *D. brevipes* is ovoviviparous and reproduces parthenogenetically in most areas, including Hawaii and Jamaica (Beardsley, 1965; Seether et al., 2005). It is believed to be of New World origin (Seether, 2002).

Molecular methods have been used successfully to determine the invasion history of invasive taxa (Downie, 2002; Facon et al., 2003; Cognato et al., 2005). Phylogenetic analyses suggested that the source of *D. brevipes* in Hawaii was likely to be the mealybug lineage that lives predominantly in Brazil, Thailand, and the Philippines, according to the sequences of mitochondrial cytochrome c oxidase subunit I (COI) downloaded from GenBank. However, phylogenetic analyses also showed that PPMs from China are of different geographic origin to the samples from Hawaii (He et al., 2012).

Owing to the difficulty of finding a regular pattern of distribution from a large number of cultivated crop varieties, parthenogenetic and wingless pests on crops represent an alternative way to investigate historical routes of dispersal. To reveal the history of introduction of *D. brevipes* in the cultivation of pineapple in China, *D. brevipes* samples were collected from main pineapple production regions in China and Thailand and their mitochondrial *COI* sequences were analyzed. Furthermore, the homologous sequences of *D. brevipes COI* deposited in GenBank from Brazil, Thailand, and the Philippines were compared.

MATERIAL AND METHODS

Processing of mealybugs

PPMs from *A. comosus* were obtained from 14 counties across five provinces in China, and a sample of PPM from Hawaii, USA, was also included. In addition, *Dysmicoccus*

neobrevipes Beardsley and *Planococcus minor* (Maskell) from China were used as outgroups in data analysis. Samples were collected in 2010-2014 (Table 1).

Table 1. Collection information of samples of *Dysmicoccus brevipes* and outgroup species.

Population name	Host species	Collection information
Dysmicoccus brevipes		
Hawaii	*Ananas comosus*	USA; Hawaii. Nov. 2010; Coll. D.M. Sether
Nanning	*Ananas comosus*	Nanning, China. May 2014; Coll. Y.B. He
Jinghong	*Ananas comosus*	Jinghong, China. Jul. 2010; Coll. Y.B. He
Xuwen	*Ananas comosus*	Xuwen, China. Oct. 2010; Coll. Y.B. He
Zhangzhou	*Ananas comosus*	Zhangzhou, China. Jul. 2010; Coll. Y.B. He
Leizhou	*Ananas comosus*	Leizhou, China. Oct. 2010; Coll. Y.B. He
Qionghai	*Ananas comosus*	Qionghai, China. Mar. 2014; Coll. Y.B. He
Wanning	*Ananas comosus*	Wanning, China. Mar. 2014; Coll. Y.B. He
Lampang 1	*Ananas comosus*	Lampang, Thailand. Apr. 2014; Coll. Y.B. He
Lampang 2	*Ananas comosus*	Lampang, Thailand. Apr. 2014; Coll. Y.B. He
Dysmicoccus neobrevipes	*Agave sisalana*	Zhanjiang, China. Sep. 2010; Coll. Y.B. He
Planococcus minor	*Mangifera indica*	Zhanjiang, China. Jul. 2010; Coll. Y.B. He

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

Samples were rinsed with double distilled water and then air dried. All specimens were examined under a microscope for the presence of parasitoids. Total DNA was extracted from single parasitoid-free adult females using the TIANamp Genomic DNA kit (Tiangen Biotech, Beijing, China), following the manufacturer protocol.

Template preparation and DNA manipulation

A 649-bp region of the mitochondrial *COI* gene was amplified with the primers F: 5'-CCTTCAACTAATCATAAAAATATTAG-3' and R: 5'-TAAACTTCTGGATGTCCAAA AAATCA-3' (Park et al., 2010). PCR amplification was performed in 25-µL reaction volumes with 3 µL template DNA. The PCR mix contained 2.5 µL 10X Taq reaction buffer (Promega, Madison, WI, USA), 0.5 U Taq DNA polymerase (Promega), 3.5 mM $MgCl_2$, and 15 pM of each primer. PCR conditions were as follows: 95°C for 3 min, followed by 34 cycles of 95°C for 30 s, 55°C for 45 s, 72°C for 1.5 min, and a final extension of 5 min at 72°C.

Each PCR product (5 µL) was 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. To achieve the whole ITS sequence, new primers were designed based on the intermediate sequence. Amplification products were purified and sequenced by Invitrogen Biotechnology (Shanghai, China) on both strands using PCR primers. Sequences of all *COI* haplotypes of PPM have been deposited in GenBank under accession No.: JN128960-JN128963; and those of *D. neobrevipes* and *P. minor* were JN128957 and JF965419, respectively. The homologous *COI* sequences of PPM from Brazil, Thailand, and the Philippines were obtained from GenBank with accession No. KJ530600-KJ530601, HM474137/HM474139, and HM474142-HM474144.

Data analysis

Sequences were aligned using ClustalX (version 2.0) and unique haplotypes were identified with Arlequin (version 3.5). Descriptive statistics (number of variable sites, number of haplotypes, haplotype diversity, nucleotide diversity, average number of nucleotide differences between haplotypes) were generated using DNASP (version 5.0), and F_{ST} was calculated using Arlequin version 3.5 (Excoffier et al., 2005).

The genealogical relationships based on 480-bp *COI* sequences of PPMs were studied by constructing a network based on the statistical parsimony method described by Templeton et al. (1992), using the software TCS 1.20 (Clement et al., 2000). A network can be more appropriate at depicting intraspecific gene genealogies than a bifurcating tree because of the potential for extinct ancestral nodes and multifurcating relationships (Posada and Crandall, 2001).

Phylogenetic analyses were performed by maximum parsimony (MP) and neighbor joining (NJ) analysis using PAUP* 4.10 (Swofford, 2003). Incongruence among data partitions was analyzed using a partition homogeneity test with 1000 replications as implemented in PAUP*. MP and NJ analyses used the heuristic search option with tree-bisectionreconnection branch swapping, collapsing zero-length branches, and equal weighting of all characters. MP bootstrap support was calculated using 1000 replicates.

RESULTS

D. brevipes was found to have a simple genetic structure, and 1-3 haplotypes of PPM were found in many populations (Table 2). According to the homologous sequences, TH1 from Thailand was the same as HI from Hawaii.

Table 2. Distribution frequencies of different haplotypes in geographical populations of *Dysmicoccus brevipes*.

Samples		Number of samples per population	Haplotypes			
Country	Population name		HI/TH1	CN1	CN2/TH2	CN3/TH3
USA	Hawaii	30	30			
China	Nanning	30		30		
	Jinghong	30		30		
	Xuwen	30		30		
	Zhangzhou	30		24	6	
	Leizhou	46		30	12	4
	Qionghai	54			48	6
	Wanning	92		25	25	42
Thailand	Lampang 1	30			30	
	Lampang 2	30			21	9
	HM474137/HM474139	Unknown	Unknown			

PPMs from Thailand and China were of three haplotypes, of which two were identical and one differed. The presence of two shared haplotypes suggests that there is a close relationship between pineapple cultivation in Thailand and China.

Haplotypes of *D. brevipes* clustered into three major clades based on the COI sequences. The first clade consisted of CN1, PHI, and CN2/TH2, the second clade included BR1, BR2, and HI/TH1, and another clade from China and Thailand was CN3/TH3 (Figures 1 and 2).

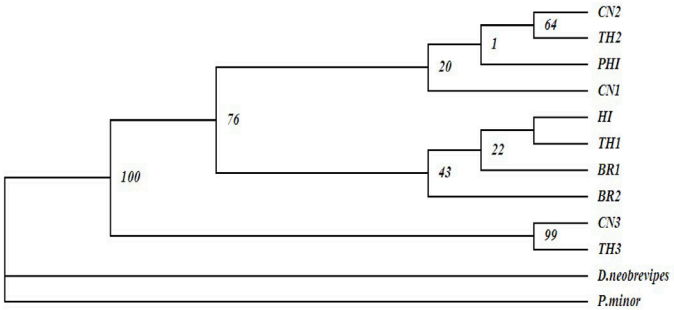

Figure 1. Maximal parsimonious tree of 10 haplotypes of *Dysmicoccus brevipes.* The PAUP 4.10 software was used for the analysis. Sequences of BR1, BR2, PHI, and TH1 were downloaded from GenBank. *D. neobrevipes* and *P. minor* were included as outgroups based on the 480-bp *COI* sequences.

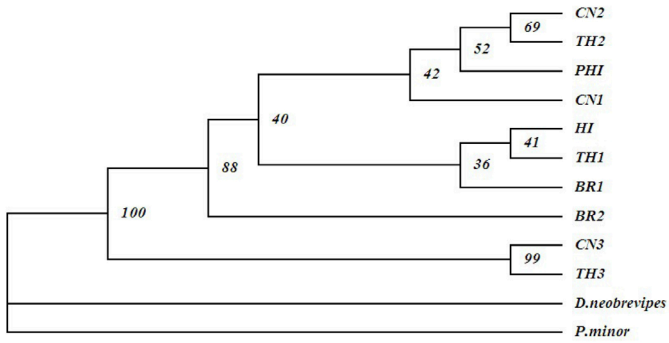

Figure 2. Neighbor joining tree of 10 haplotypes of *Dysmicoccus brevipes.* The PAUP 4.10 software was used for the analysis. Sequences of BR1, BR2, PHI, and TH1 were downloaded from GenBank. *D. neobrevipes* and *P. minor* were included as outgroups based on the 480-bp *COI* sequences.

The genetic distance between BR1, BR2, HI/TH1, CN1, PHI, and CN2/TH2 haplotypes was less than 0.006, which is far lower than the genetic distance between CN3/TH3. The genetic distance between CN3/TH3 and other haplotypes ranged from 0.020 to 0.024, which is far lower than their genetic distances from the related species, *D. neobrevipes* (Table 3).

Table 3. Genetic distances among different haplotypes of *Dysmicoccus brevipes* and *Dysmicoccus neobrevipes*.

	CN3	TH3	CN2	TH2	PHI	CN1	HI	TH1	BR1	*D. neobrevipes*	*P. minor*
CN3	-										
TH3	0.000	-									
CN2	0.022	0.022	-								
TH2	0.022	0.022	0.000	-							
PHI	0.020	0.02	0.002	0.002	-						
CN1	0.024	0.024	0.006	0.006	0.004	-					
HI	0.022	0.022	0.004	0.004	0.002	0.006	-				
TH1	0.022	0.022	0.004	0.004	0.002	0.006	0.000	-			
BR1	0.024	0.024	0.006	0.006	0.004	0.008	0.002	0.002	-		
BR2	0.020	0.020	0.006	0.006	0.004	0.008	0.002	0.002	0.004	-	
D. neobrevipes	0.108	0.108	0.118	0.118	0.115	0.115	0.119	0.119	0.116	0.115	-
P. minor	0.204	0.204	0.188	0.188	0.185	0.185	0.186	0.186	0.182	0.181	0.182

Sequences of BR1, BR2, PHI, and TH1 were downloaded from GenBank.

This indicates that there are close genetic relationships among populations represented by the BR1, BR2, HI/TH1, CN1, PHI, and CN2/TH2 haplotypes. The population represented by CN3/TH3 from China and Thailand diverged substantially from other populations.

DISCUSSION

Originating in the Amazon Basin in South America, *A. comosus* was introduced to Southeast Asia by the Portuguese in the 1550s (Institute of Fruit Tree Research, GDAAS, 1987). Pineapple was not cultivated commercially in large areas of south China until the late 19th and early 20th century (Zhao and Shen, 1987). Although there was not a explicit detailed scheme for that history, probably with the same as 'Smooth Cayenne' 300-400 years later (Figure 3). Distribution of the cultivar 'Smooth Cayenne' illustrates how the pineapple was introduced and the process of its cultivation around the world.

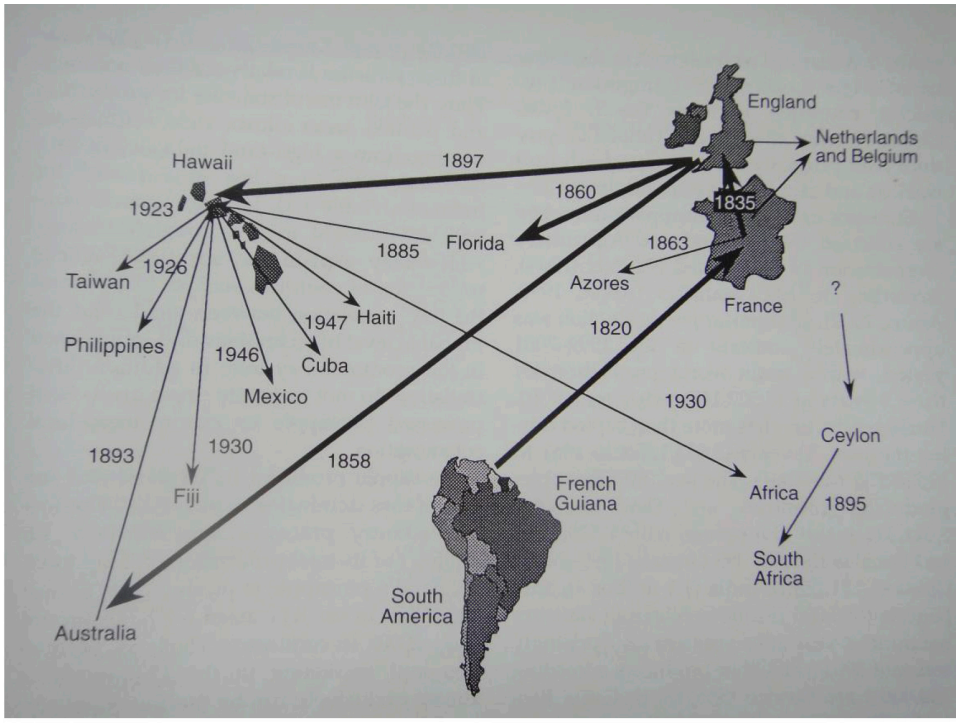

Figure 3. Distribution of the 'Smooth Cayenne' cultivar (Collins, 1951).

PPM is ovoviviparous and reproduces parthenogenetically in most areas as a wingless insect, and the female mealybug is normally distributed whilst hiding in the seedlings (He et al., 2012). Phylogenetic analyses suggest that there are close genetic relationships between PPM populations from Hawaii, Brazil, the Philippines, and from some resources from Thailand and China, and that they all probably originate from South America. It is thought that most PPMs in China were introduced from South America by way of Southeast Asia, being accompanied by the pineapple seedling. This is consistent with the history of the introduction

of the pineapple and the process of its cultivation around the world.

It is difficult to speculate on the origin of a crop because of its frequent natural or artificial hybridization and the resulting complex genotypes. To a large extent, as a parasite, PPM is an appropriate alternate that can reveal the history of the introduction and cultivation of pineapple, because of its parthenogenesis and single genotype.

According to our result, some PPMs represented by Haplotype-WN from Wanning of China, and Lampang of Thailand differ greatly from populations in Hawaii, Brazil, the Philippines, and other samples from Thailand and China. PPMs from Southeast Asia have at least two origins, one is South America, and the other is Southeast Asia itself or elsewhere. In addition to South America, pineapples in Southeast Asia might also have another origin.

Conflicts of interest

The authors declare no conflict of interest

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