Genetic Variation of Cassava Mealybug, *Phenacoccus manihoti* (Hemiptera: Pseudococcidae), Based on DNA Sequences from Mitochondrial and Nuclear Genes

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Abstract

The present study aimed to investigate the genetic variation and genetic structure of the *Phenacoccus manihoti* Matile-Ferrero, one of the most serious insect pests of cassava worldwide, in populations in Thailand, using mitochondrial and nuclear DNA sequence based analysis. The samples of *P. manihoti* were collected from 28 major cassava-growing areas within 18 provinces in Thailand. Our field survey results showed that the northeastern and eastern regions of Thailand were widely and highly infested with *P. manihoti*. Phylogenetic analysis revealed 2 mitochondrial clades and a single nuclear clade, which corresponded to low genetic variability. This suggests that *P. manihoti* has a high potential to spread aggressively throughout the cassava-growing areas in Thailand that in which it was first found in 2008. In addition, the generally low genetic divergence observed may be due to the highly prevalent parthenogenetic reproduction of this insect pest species. Further research is therefore necessary to develop proportional prevention and surveillance programs for early detection and rapid response. In addition, the genetic structure and variability of *P. manihoti* populations from neighboring countries should be studied.

Keywords: Genetic variation, cassava mealybug, *Phenacoccus manihoti*, DNA sequence

Introduction

The cassava or pink cassava mealybug, *Phenacoccus manihoti* Matile-Ferrero (Hemiptera: Pseudococcidae), is a serious insect pest that causes damage to a wide range of cassava varieties (*Manihot esculenta*) worldwide [1,2]. *P. manihoti* populations peak during the dry season [1-3]. *P. manihoti* is indigenous to South America [4], but it has been introduced to sub-Saharan Africa, and is well established throughout this region [5]. In Asia, this mealybug species was accidentally introduced to Thailand [2]. Afterwards, the pest has spread aggressively throughout cassava-growing areas in Thailand and neighboring countries such as Laos and Vietnam [2,6-8].

Many plant species can support *P. manihoti*, but only cassava is known to experience significant damage by this insect pest [2,9]. *P. manihoti* causes severe distortion of terminal shoots, curling of leaves, reduced internodes, stunting, and weakening of stems used for crop propagation [2]. Almost 150 cassava cultivars are known to be susceptible to *P. manihoti* [7]. In addition, no cultivars of cassava are known to be fully resistant [10]. Interestingly, *P. manihoti* is a parthenogenetic insect, producing only female offspring [5]. Therefore, a single adult female may be sufficient to start an outbreak [2]. Under optimal conditions, individual adult females of *P. manihoti* can lay between 200 - 600 eggs within ovisacs on the undersides of cassava leaves and around apical and lateral buds [11]. Subsequently, eggs hatch into mobile crawlers, which can spread over the plant or passively disperse to neighboring plants by wind [2,11].

Recently, the development of molecular markers has become widely used to address genetic relationships, phylogenies, population dynamics, and gene mapping in many insects [12-14]. The use of microsatellites, random amplified polymorphism DNA (RAPD), express sequence tags (EST), amplified fragment length polymorphism (AFLP), and mitochondrial DNA (mtDNA) fingerprinting in entomological research has increased our knowledge of insect ecology and evolution at the molecular level [15].

The study of genetic diversity has become essential to understand interactions between insect genotypes and environment [14,16]. In addition, the genetic diversity of insect pests affecting agriculture has been more clearly verified through use of molecular DNA markers.

mtDNA is one of the most popular molecular markers used to address population genetics and evolution of insects [17]. mtDNA has a relative high mutation rate [13,17-20], resulting in significant variation in mtDNA sequences [13,20]. For instance, the mtDNA cytochrome oxidase I (*COI*) gene has been used to study intraspecific variation of insect pests such as whitefly [21-24] and mealybug [14]. Although *COI* region is a protein coding gene, the rate of evolution of this region is 3 times greater than former barcoding genes, such as 12S and 16S ribosomal DNA, from nuclear genomes [25-27].

However, the use of only mtDNA as a genetic marker in molecular ecology and phylogeographic studies possibly leads to misinterpretation and may yield an incomplete evolutionary history of the target species [28,29]. This may be due to mitochondrial recombination in population levels [30], mitochondrial introgression [31-33], and the indirect selection caused by symbionts [34]. Thus, the combined used of mitochondrial and nuclear DNA, as opposed to the sole usage of mtDNA markers, could allow researchers to answer more complicated evolutionary questions about target organisms [35].

Insecticide resistance is the result of an increase in the ability of the individual of an insect species to survive insecticide application [36]. Resistance alleles may arise and spread in insect populations and to other populations with variable success, depending on factors such as genetic variability, gene flow, population size, and environmental factors [37]. Lenormand [38] reported that the rate of development of insecticide resistance may be influenced by the gene flow between treated and untreated populations. This report is in close agreement with Kazachkova *et al*. [37], who investigated the genetic diversity of the pollen beetle (*Meligethes aeneus*) population in Sweden. They showed that the level of genetic variation within insecticide resistant populations were very high. They also found that there appears to be a high rate of gene flow between populations. Therefore, the aims of this study are to investigate the genetic structure and diversity of *P. manihoti*, an aggressive insect pest of cassava, in populations from different regions in Thailand by analyzing variation in a partial sequence of the mitochondrial cytochrome oxidase I (*COI*) gene and the nuclear internal transcribed spacer 1 (*ITS1*) gene.

Materials and methods

Sample collection

P. manihoti, samples were collected from 28 major cassava-growing areas in 18 of the 77 provinces in northeastern, eastern, central, and western Thailand. Each individual insect sample was placed in a 1.5 ml micro-centrifuge tube containing 95 % ethanol. Furthermore, the geographical position of each collecting site was determined using GPS. Species identification of insect specimens was then confirmed using the identification key of Parsa *et al*. [2].

DNA extraction and PCR amplification

Genomic DNA from each individual *P. manihoti* per collecting site (28 individuals in total) was extracted using DNeasy® Blood & Tissue kit (Qiagen, Germantown, MD, US; catalog # 69504). In this study, we used the protein-coding *COI* gene and the *ITS1* gene for molecular phylogenetic analysis. Polymerase chain reaction (PCR) amplification of a gene fragment was carried out in a 50 µl final reaction volume containing 1x PCR master mix (Catalog#K0171, Fermentas Life Science), 200 nmol/l of a foreword primer (*COI*: 5'–ATAACTATACCTATYATTATTGGAAG- 3'; *ITS1*: 5'-TACACACCGCCCGTCG CTACTA-3') and a reverse primer (*COI*: 5'-TAAACTTCAGGGTGACCAAAAAATCA-3'; *ITS1*: 5'-ATGTGCGTTCG AAATGTCGATGTTCA-

3') [39], and at least 200 ng of DNA template. Thermal profiles consisted of an initial denaturation step of 94 ℃ for 3 min, followed by 35 cycles of 94 ℃ for 1 min, 48 ℃ for 1 min, and 72 ℃ for 150 s, with a final extension step of 72 ℃ for 5 min. Amplified PCR products were then purified using QIAquick® Gel Extraction kit (Qiagen, Germantown, MD, US; catalog # 28740), and direct sequenced by AITbiotech Pte Ltd (Singapore).

Table 1 Collection of *P. manihoti* from major cassava-growing areas (northeastern, eastern, central, and western) in Thailand.

| No.	Location	Latitude	Longitude	Date
1	Sakon Nakhon1 (Phuphan)	16° 53.034 N	103° 56.857 E	10/12/2013
2	Sakon Nakhon2 (Tao Ngoi)	16° 56.221 N	104° 10.236 E	10/12/2013
3	Mukdahan1 (Mueang)	16° 41.682 N	104° 30.413 E	09/03/2014
4	Mukdahan2 (Nikhum Kham Soi)	16° 25.661 N	104° 35.633 E	09/03/2014
5	Nakhon Phanom (Mueang)	16° 57.086 N	104° 42.127 E	09/03/2014
6	Roi Et (Ponthong)	16° 18.777 N	103° 58.888 E	10/11/2013
7	Maha Sarakham (Wapi Pathum)	15° 54.721 N	103° 27.367 E	10/03/2014
8	Khon Kaen (Ban Pai)	16° 02.786 N	102° 53.861 E	10/11/2013
9	Chaiyaphum1 (Thapsathit)	15° 39.399 N	101° 29.322 E	10/09/2013
10	Chaiyaphum2 (Chatturat)	15° 27.518 N	101° 49.588 E	10/09/2013
11	Nakhon Ratchasima1 (Pha Thong Kham)	15° 22.444 N	101° 54.086 E	10/09/2013
12	Nakhon Ratchasima2 (Mueang)	15° 02.428 N	102° 11.718 E	10/09/2013
13	Nakhon Ratchasima3 (Nonthai)	15° 08.972 N	102° 05.893 E	10/09/2013
14	Buriram1 (Satuk)	15° 14.874 N	103° 15.166 E	10/03/2014
15	Buriram2 (Lumpaimas)	15° 01.229 N	102° 49.185 E	10/03/2014
16	Prachinburi (Kabinburi)	14° 01.591 N	101° 40.542 E	11/03/2014
17	Kanchanaburi1 (Thong Pha Phum)	14° 43.724 N	098° 34.958 E	19/10/2013
18	Kanchanaburi2 (Sai Yok)	14° 06.258 N	099° 13.301 E	18/10/2013
19	Chonburi1 (Sattahip)	12° 57.481 N	100° 58.395 E	14/05/2014
20	Chonburi2 (Pattaya)	12° 57.481 N	100° 58.395 E	14/05/2014
21	Rayong1 (Mueang)	12° 41.127 N	101° 13.495 E	16/11/2013
22	Rayong2 (Mueang)	12° 42.434 N	101° 11.236 E	15/05/2014
23	Rayong3 (Klaeng)	12° 45.505 N	101° 38.225 E	15/05/2014
24	Chanthaburi (Kaeng Hang Maeo)	12° 59.209 N	101° 53.538 E	15/05/2014
25	Saraburi (Kaeng Khoi)	14° 41.120 N	100° 05.521 E	10/08/2013
26	Uthai Thani (Mueang)	15° 22.280 N	100° 02.583 E	21/05/2014
27	Chainat (Mueang)	15° 14.189 N	100° 12.249 E	21/05/2014
28	Phichit (Sam Ngam)	16° 25.626 N	100° 08.658 E	04/10/2013
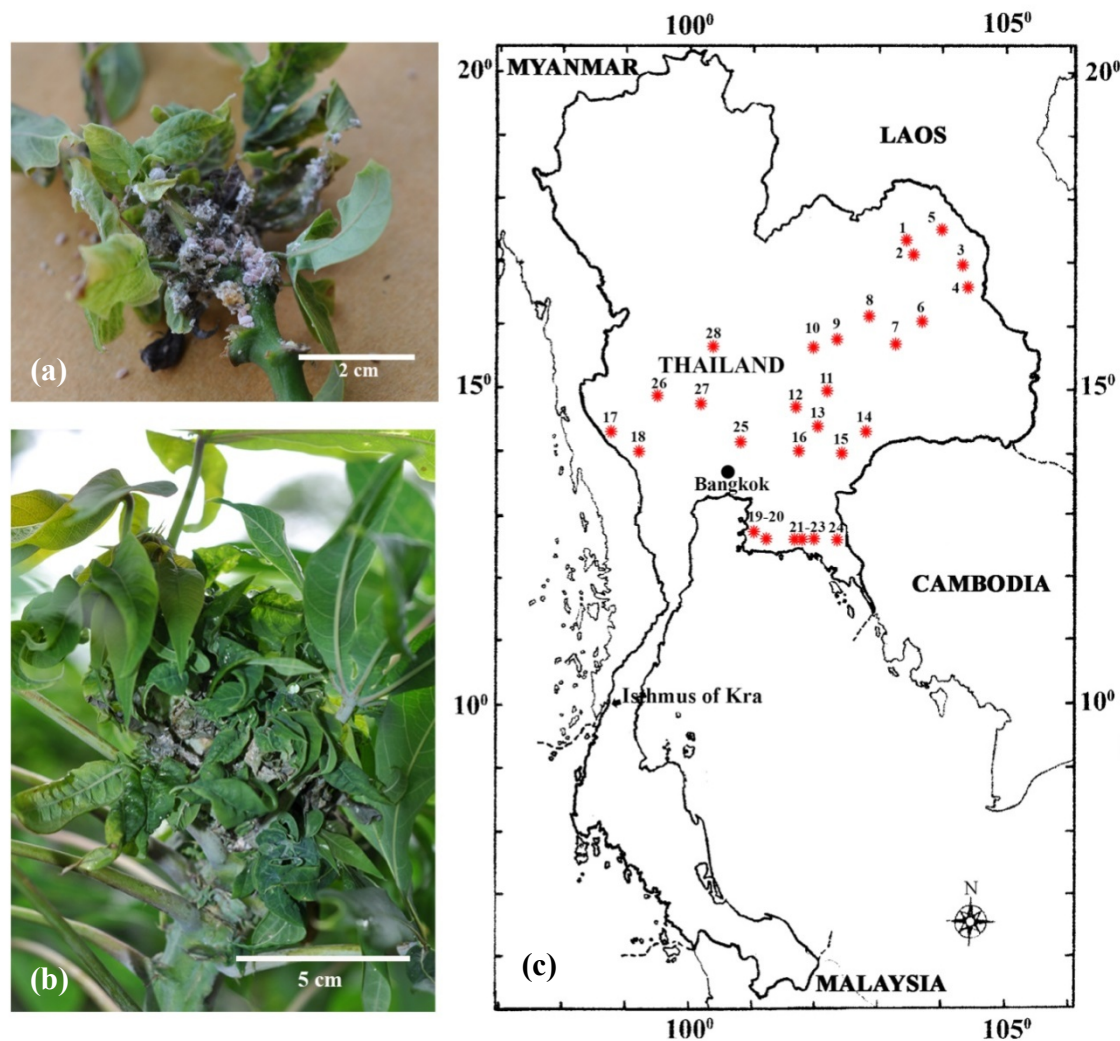
Figure 1 (a) Heavy infestations of *P. manihoti* and associated terminal shoot distortion. (b) Stem deformation of cassava associated with *P. manihoti* infestations. (c) *P. manihoti* collection sites in Thailand and numbers corresponding to those in **Table 1**.

Sequence analysis and Phylogenetic reconstruction

Partial DNA sequences were initially aligned and edited using MEGA6 v6.06 [40]. The extent of genetic variation was determined as the average number of nucleotide differences (k), number of polymorphic sites (s), haplotype diversity (h) and nucleotide diversity (π), by using DNAsp v5.0 program [41].

The Maximum Likelihood (ML) and Bayesian Inference (BI) methods were used to reconstruct the phylogenetic relationship among *COI* and *ITS1* haplotypes. The program Kakusan4 {[42]; with maximum likelihoods calculated in Treefinder: [43]} was used to estimate the best-fit models of nucleotide substitution, as adjudicated by the Akaike information criterion (AIC: [44]) implemented for ML and by the Bayesian information criterion (BIC: [45]) for BI. The ML analysis was performed on Treefinder [43], using the likelihood-ratchet method, with 1000 bootstrap replicates to estimate branch confidence

values. Tree topologies with bootstrap values (bs) of 70 % or greater were regarded as sufficiently resolved. The BI analysis was performed with MrBayes v3.1 [46], which employs a Metropolis-coupled, Markov chain Monte Carlo (MC-MCMC) sampling approach. A 4-chain MC-MCMC analysis was run twice in parallel (with default heating values) for 1 million generations, starting with random trees, and trees were collected every 100 generations [46]. The log-likelihood values of the sample points were plotted against the generation time, and 25 % of the generations were discarded as ''burn-in'' samples. The remaining trees were used to estimate consensus tree topology, bipartition posterior probability (bpp), and branch length [46]. Bipartition posterior probability that was 0.95 or greater was considered as significantly supporting [47].

Results and discussion

The invasive distribution of *P. manihoti* in Thailand

In this study, we surveyed the invasion of *P. manihoti* and collected samples throughout Thailand. We found that the northeastern and eastern regions of Thailand are widely and highly infested with *P. manihoti* (**Figure 1** and **Table 1**). Interestingly, our field survey results found no *P. manihoti* infestations in the northern and southern regions of Thailand. These results suggest *P. manihoti* distribution in Thailand is likely to be limited by cold weather in the north and high rainfall in the south. Parsa *et al*. [2] estimated the climatic suitability for *P. manihoti* regional spread using a CLIMEX distribution model. They showed that the model suggests *P. manihoti* is likely to be limited by cold stress across Vietnam's northern regions and in the entire Guangxi province of China, and by high rainfall across the wet tropics in Indonesia and the Philippines. According to the results of experiments of laboratory conditions at 25 °C, the development optimum is about 27 °C [11], whereas significant mortality occurs below 15 °C and above 33 °C [48]. Moreover, rainfall is one of the key factors that suppress *P. manihoti* population growth, not only by increasing mechanical mortality [49], but also by reducing cassava's suitability as a host [10, 50]. Dry regions, years, and seasons therefore favor outbreaks of this insect pest [10]. Thus, the management decision to control the *P. manihoti* invasion in Thailand may first be of concern in hot and dry climatic regions such as the northeastern, eastern, and western areas.

Sequence analysis

Under optimal PCR conditions, we obtained DNA fragments containing 419 base pairs (bp) of the *COI* gene and 604 base pairs (bp) of *ITS1* from all 28 *P. manihoti* samples. All PCR products of *COI* of samples from all collecting localities were high in A+T content (average of 71.48 %, calculated from the total amounts of nucleotides in the obtained sequences). The nucleotide composition of *COI* was detected to be A=33.1, T=47.7, G=5.5, and C=13.7 %, whereas the *ITS1* gene composition was A=24.4, T=25.0, G=23.3, and C=27.3 %. Variance was detected due to substitutions (transversion and transition) without any indels. Multi-alignment and pair-wise sequence comparisons showed a total of 23 polymorphic sites in *COI* gene sequences and 9 variable sites in *ITS1* gene sequences. The frequency of transversion mutations of *COI* and *ITS1* gene sequences were 71.43 and 88.89 %, respectively. Transition mutations of *COI* and *ITS1* gene sequences were 28.57 and 11.11 %, respectively. Our polymorphic site analysis results supported the observation that mitochondrial *COI* gene showed higher mutation rates [13,17-20]. Thus, the mitochondrial *COI* gene might be suitable for the intraspecific variation analysis of insect pests.

Seven *COI*-haplotypes and 6 *ITS*-haplotypes of *P. manihoti* were detected in this study. The total number of h inferred from *COI* and *ITS1* gene sequences were 0.653 and 0.648, respectively. The π of all samples for *COI* sequences was 0.0072 (range from 0.00250 - 0.01989), whereas the π of all samples for *ITS1* sequences was found to be 0.00287 (range from 0.00166 - 0.00497). A lower average number of k inferred from *ITS1* gene sequences (1.090) was found when compared to *COI* gene sequences (2.608). A summary of the molecular diversity indices of *COI* and *ITS1* gene sequences of *P. manihoti* are shown in **Table 2**.

Table 2 Summary of molecular diversity indices of *COI* and *ITS1*; sample size (*n*), number of haplotypes (*No.*), number of polymorphic (segregating) sites (*S*), average number of nucleotide differences (*k*), haplotype diversity (*h*) with standard deviation (SD) and nucleotide diversity (π).

Gene	Region	*n*	*No.*	*S*	*k*	*h* (±SD)	Π
COI	Northeastern	15	4	4	1.048	0.552 (0.137)	0.00250
	Central	5	4	6	2.900	0.900 (0.161)	0.00697
	Eastern	6	3	25	8.333	0.600 (0.215)	0.01989
	Western	2	2	2	2.000	1.000 (0.500)	0.00477
	All samples	28	7	29	3.000	0.653 (0.091)	0.00721
ITS1	Northeastern	15	6	5	1.467	0.829 (0.064)	0.00243
	Central	5	4	7	3.000	0.900 (0.161)	0.00497
	Eastern	6	5	4	1.800	0.933 (0.122)	0.00298
	Western	2	2	1	1.000	1.000 (0.500)	0.00166
	All samples	28	6	11	1.735	0.648 (0.036)	0.00287

Phylogenetic analysis

Phylogenetic analysis demonstrated that there were 2 major mitochondrial *COI* clades (mt*COI* clade) with low bootstrap value for ML and probability for BI (**Figure 2a**). The mt*COI* clade 1 consisted of the samples from Chainat, Saraburi, Chaiyaphum1, Nakhon Ratchasima1, Kanchanaburi2, Mukdahan2, and Chanthaburi, while all other samples were in the mt*COI* clade 2. A single nuclear *ITS1* clade was detected (**Figure 2b**). These results closely agreed with the sequence analysis results, which showed low genetic diversity indices in all samples. Bouga *et al.* [17] reported no variation among *Marchalina hellenica* (Hemiptera: Margarodidae) populations in Turkey based on mtDNA *COI* gene. They suggested that the low genetic diversity observed was expected because of all of the sequencing data obtained from the *COI* segment, using barcode primers applied for the identification of species.

Interestingly, Rosas-García *et al.* [14] investigated genetic differentiation among pink hibiscus mealybug, *Maconellicoccus hirsutus*, populations living on different host plants, using amplified fragment length polymorphism (AFLP). They found a high differentiation in *M. hirsutus* populations among the host plant species, supported by a Bayesian analysis, which revealed an *M. hirsutus* population grouping robustness according to their host plants. They concluded that genetic variation among populations of *M. hirsutus* is caused by host plants, not by geographic distance. These findings are in close agreement with those of Singh *et al.* [51], which showed that the genetic similarity of solenopsis mealybugs (*Phenacoccus solenopsis*) also depended on host plants.

The low observed genetic diversity of *P. manihoti* populations in this study suggests they have a high potential to become invasive throughout cassava-growing areas. This would be facilitated by the high rates of parthenogenetic reproduction of this insect pest species [5]. Hence, a single immature or adult insect may be sufficient to start an outbreak [2]. In addition, this mealybug species has only been known to occur in Thailand since 2008 [2]. Therefore, further biological research, including monitoring and surveillance programs, into this species are required.
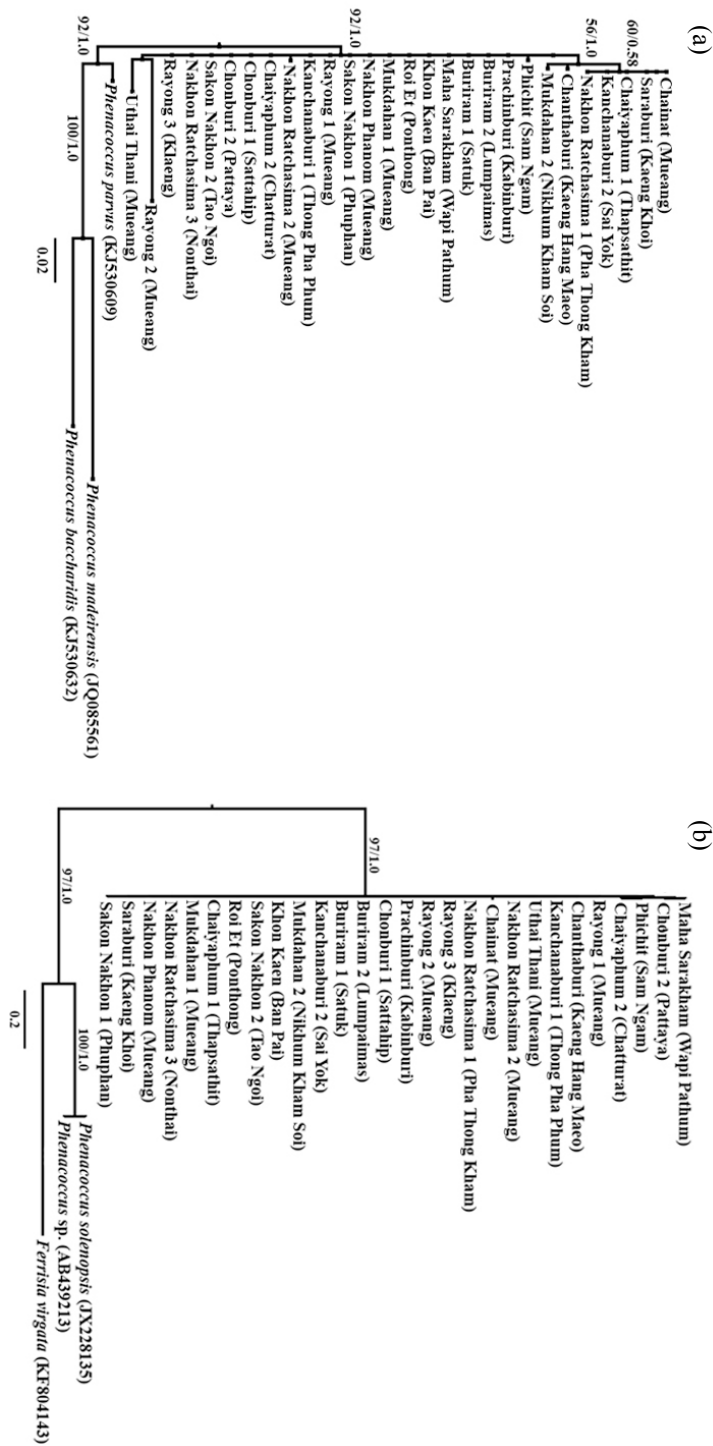
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http://wjst.wu.ac.th

Figure 2 Phylogenetic relationships of *P. manihoti* and outgroups based on Bayesian inference analysis (BI) of *COI* (a) and *ITS1* (b).

Conclusions

We determined the genetic variation of cassava mealybug (*P. manihoti*) populations collected from 28 major cassava-growing areas within 18 provinces in Thailand using mitochondrial and nuclear DNA sequence based analysis. Although 7 mitochondrial *COI* and 6 nuclear *ITS1* haplotypes were found, low genetic diversity indices were detected. The low genetic variation among *P. manihoti* populations was in close agreement with phylogenetic analysis, which showed 2 mitochondrial clades and a single nuclear clade. These results suggested a high potential for population reproduction in this species. However, comprehensive molecular genetic analyses, including samples from neighboring countries of Thailand, should be conducted to confirm the population variability of this serious insect pest of cassava.

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