

Genetic variation among the geographic population of Cassava mealybug *Phenacoccus manihoti* (Hemiptera: Pseudococcidae) in Indonesia inferred from mitochondrial COI gene sequence

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Abstract. Nopriawansyah N, Rauf A, Kusumah YM, Nurmansyah A, Koesmaryono Y. 2019. Genetic variation among the geographic population of Cassava mealybug *Phenacoccus manihoti* (Hemiptera: Pseudococcidae) in Indonesia inferred from mitochondrial COI gene sequence. *Biodiversitas* 20: 2685-2692. Cassava mealybug, *Phenacoccus manihoti* Matile-Ferrero (Hemiptera: Pseudococcidae) is an invasive pest native to South America. Research was conducted with the objective to study genetic variation of cassava mealybug from different geographic areas in Indonesia. Thirty-six samples of cassava mealybugs were collected from eight different population locations. Genomic DNA was extracted from individual specimens of *P. manihoti* using *gSYNC DNA Extraction Kit* from Geneaid Lot No. FB221707. PCR amplification using primary COI gene showed the target position as long as 518 bp. Analysis of homology from eight populations revealed eight haplotypes in Indonesia with different variable sites. Genetic distance analysis was done by calculating base pair differences in each population. The percentage of genetic distance between populations was 0.0000% -0.00409%. The results of the analyst using the Kimura-2 parameter showed that the population was divided into 2 clusters. The highest haplotype (*Hd*) diversity value was 0.8333, while the lowest was zero (*Hd* = 0). The highest nucleotide diversity (π) was 0.00225 and the lowest was 0.000000. The genetic distance and nucleotide diversity were low because *P. manihoti* was thelytoky parthenogenesis.

Keywords: Cassava mealybug, COI, genetic diversity, *Phenacoccus manihoti*

INTRODUCTION

The cassava mealybug *Phenacoccus manihoti* Matile-Ferrero (Hemiptera: Pseudococcidae), is an invasive alien pest that originates from South America (Cox & Williams 1981). It devastated cassava when inadvertently introduced to Congo in early 1970s and from there it spread to rest of the cassava growing areas in the continent of Africa. In the absence of its natural enemies, the mealybug spread rapidly across tropical Africa (Neuenschwander 2003). In South America, *P. manihoti* is present only in restricted areas of Paraguay, Brazil, and Bolivia (Williams & Granara de Willink, 1992). In 2008, *P. manihoti* was discovered in Asia for the first time, damaging cassava in Thailand (Winotai et al, 2010). The cassava mealybugs have also been recorded in Malaysia (Sartiami et al. 2015). In Indonesia, this pest was first detected in Bogor (West Java) in 2010 (Muniappan et al. 2011). Based on the field survey in 2016-2017, *P. manihoti* had already been widespread in Java, Lampung, NTB and NTT (Abduchalek et al. 2017; Wyckhuys et al. 2018).

This mealybug species has been recognized as the main important pest in the world where there are cassava plantations. *P. manihoti* reproduces through thelytoky parthenogenesis, which all the offspring produced are

female, hence one single mealybug can reproduce and in the abundant population, it can cause pests explosion in the plantations (Catalayud & Ru 2006). The attack of *P. manihoti* in cassava plants generally occurs in the shoots. At a very high population density, and as it is usually the case in dry season, the attack caused the shoots to curl, book segment to shorten, and plant to become stunted (Wyckhuys et al. 2018). Since the 1970s, *P. manihoti* attacks on cassava plantations in Africa has caused severe damage and huge loss of yield and even raised problems of hunger for local people (Parsa et al. 2012). Severe attacks can cause about 80% loss of results as happened in Africa (Nwanze 1982) and 40-50% in Asia (Wyckhuys et al. 2014). Therefore, *P. manihoti* can threaten cassava production and food security in affected areas (Yonow et al. 2017).

The information on the genetic aspects of the geographic variation in an insect pest can provide important biological information for deploying aphid-resistant cultivars, and developing alternative chemicals for control strategies (Xu et al., 2011). The high degree of morphological similarity among different mealybug taxa is a major problem for *P. manihoti* control and assessment of its systematic biology or their populations. The morphological examination that is time-consuming and can

be conducted only in the appropriate development stage (mostly on adult females) requires a high level of expertise and can not always distinguish among taxa especially with a high degree of similarity (Malausa 2011).

Molecular techniques are one way to find out the ecology and genetics of insects. DNA analysis can provide a more detailed evaluation of analysis of biotype, colonization, dispersal, diversity between species and intraspecies, insect interactions, and morphologically similar species, and is one of the most popular molecular markers used for population genetics and insect evolution (Hoy 1994). Mitochondrial DNA has an evolutionary rate of 1 to 10 times faster than nuclear DNA (Avisé et al. 1987). mtDNA is widely used to study genetic variation, population genetic structure, phylogeny, and phylogenetic relationships at various taxonomic levels (Xu et al. 2011).

Mitochondrial DNA is generally used to study genetic variation, population genetic structure, phylogeny, and phylogenetic relationship at various levels of the taxonomy (Xu et al. 2011). The most widely used mitochondrial DNA is the cytochrome c oxidase subunit 1 (CO1) gene while the most widely used marker in the analysis of genetic variation is mt COI and mt COII (Simon et al. 2006). The CO1 gene is also used to solve-how does it solve, find the correct word to use here cryptic species, synonyms, and species extinction (Hebert et al. 2003b). Using the CO1 gene has been carried out to see characterization and identification of the genus Pseudococcidae (Malausa et al. 2011) and genus Lepidoptera (Palraju et al. 2018). In Thailand, the use of the CO1 mtDNA gene in the analysis of the genetic variation of *P. manihoti* has been reported and it found 7 haplotypes (Rattanawanee & Chongrattanameteekul 2015). The COI gene is also used to test the evolutionary pace of invasive species compared to closely-related non-

invasive species, and to see genetic diversity between invasive species (Young et al. 2018).

This study aimed to study the genetic variation of *P. manihoti* in Indonesia based on the analysis of markers of the mitochondrial DNA CO1 gene.

MATERIALS AND METHODS

Sample collection and identification of *P. manihoti*

A total of 36 *P. manihoti* individuals were collected from 8 locations (populations), namely Bangka Island, Sumatra Island (Lampung), Java Island (Cilegon, Bogor, Semarang, Pati, Malang) and Lombok. Figure 1 Location of the sampling distribution. Samples were maintained in the laboratory before identification was carried out morphologically to ensure that the sample was *P. manihoti*. Morphological identification was conducted under the Hyrox microscope using an identification key from Williams and de Willink (1992). The identified mealybugs were then collected, soaked in 95% ethanol, and used for DNA extraction.

DNA extraction

The DNA genome was extracted from individual *P. manihoti* using the gSYNC DNA Extraction Kit from Geneaid with the extraction method by the Geneaid kit protocol. The extracted DNA was visualized through 1.2% agarose gel electrophoresis method using a 1xTAE buffer (40 mM Tris-acetate, 1 mM EDTA) for 50 minutes. Agarose gel was then stained with ethidium bromide (0.5 g / ml), observed, and documented using GEL DOC under 400 nm ultraviolet light.

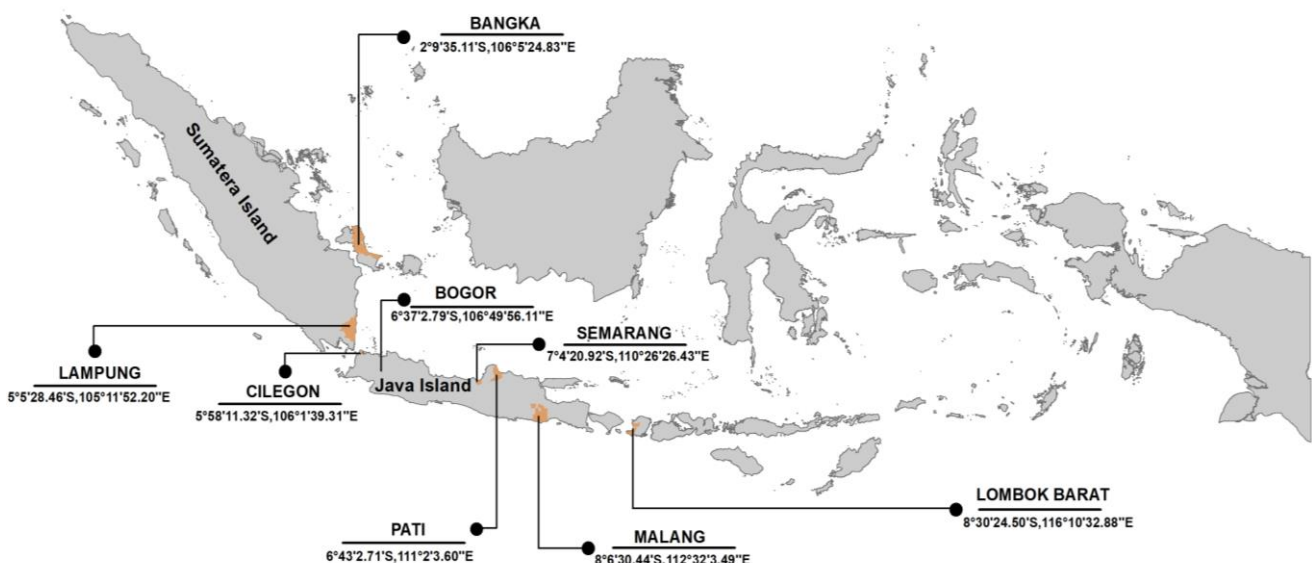


Figure 1. Map of *Phenacoccus manihoti* sampling location in Indonesia

Amplification and sequencing

DNA amplification used the GeneAmp PCR System 9700 engine. The volume of reactants was 25 µL which consisted of PCR-grade water 8.5 µL, master mix 2X KAPA Taq Extra HS with Dye 12.5 µL, 1 µL forward primer, 1 µL reverse primer and 2 µL template DNA. The pair of CO1 primers used were primer LCO1490 forward 5'ATAACTATACCTATYATTATTGGAAG'-3 and HCO 2198 reverse 5'-TAAACTTCAGGGTGACCAAAAAA TCA'3 (Rattanawanee and Chongrattanameteeikul 2015). The PCR conditions used were as follows, (i) denaturation at 95°C for 5 minutes, (ii) annealing 48°C for 30 seconds, and (iii) extension of chain (synthetic DNA) at 72°C for 5 minutes. PCR products were detected by electrophoresis on 1.2% agarose gel using TAE-1x buffer. The PCR product which showed a single band ribbon was ended for DNA sequencing at 1st BASE, Singapore.

Data analysis

Sequencing data were aligned using the MEGA Ver.6 application (Molecular Evolutionary Genetics Analysis) (Tamura et al. 2013). Analysis of population structure was done through differences in the level of distance between populations. Arlequin application (Excoffier et al. 1992). Calculation of genetic distance and reconstruction of phylogenetic trees were based on Neighbor-joining methods employing Kimura 2-parameter method which was repeated using bootstraps 1000 times. Analysis of genetic diversity was carried out for all individuals obtained at each observation location as representation of samples at each location. This analysis used the DNA Sequence Polymorphism application as a standard measure in calculating genetic diversity which included the diversity of haplotypes (*Hd*) and nucleotide diversity (π) (Rozas et al. 2003).

RESULTS AND DISCUSSION

Morphology of *Phenacoccus manihoti*

Characteristics of *P. manihoti* from the results of making slide preparations were 1.70mm long and 0.90mm wide, cerarii numbering 9-18 pairs, Circulus and oral rim tubular ducts entirely absent, anal lobe bars absent, antennae with 9 segments, claw denticles usually present, Multilocular disc pores on dorsum not present in rows across the segments, Quinquelocular pores anterior to clypeolabral shield numbering 32-68.

Characteristics of *Phenacoccus manihoti* based on gen CO1

Total DNA in *P. manihoti* samples were successfully isolated. The DNA band from the PCR products showed the position of the CO1 gene with a length of 518 bp (Figure 2). BLAST of the CO1 gene sequence showed the identity of eight populations at 99% with the query cover 97%. Multiple alignments of the CO1 gene from eight populations with a total of 36 *P. manihoti* individuals showed a conservative site of 434 (83622%) as the highest and the lowest was a parsimony site of 10 (1.92%). The

average composition of A + T nucleotide base pairs was higher (79.7%) than G + C (20.3%) in the CO1 gene (Table 1).

Homology analysis of mitochondrial CO1 Genes

Analysis of the mitochondrial DNA COI gene *P. manihoti* obtained a length of 518 base pairs. Analysis of homology from eight populations found eight haplotypes in Indonesia with different variable sites. There are seven variable sites that change, i.e., in the 9th, 10th, 12th, 509th, 510th, 511th, and 512th nucleotide sequences. The highest number of individuals in the haplotype are 13 individuals in several populations, consisting of *P. manihoti* (Bangka-1, Bangka-2, Bangka-3, Bangka-4, Bangka-5, Malang-1, Malang-2, Malang-3, Malang-4, Pati-1, Pati-2, Pati-3, Pati-4). The Lampung population has three haplotypes (Table 1). This analysis shows the existence of specific nucleotide sites between *P. manihoti* populations from Indonesia with GenBank *P. manihoti* data from China. Specific nucleotide sites were found at the 12th position with nucleotide T (Timin) characteristics for Indonesia and A (Adenine) for China (Table 2).

Genetic variation

The diversity of haplotypes (*Hd*) between populations ranges from 0.00000 to 0.83333. The highest haplotype (*Hd*) diversity value was found in Lampung population at 0.83333 while the lowest was zero (*Hd* = 0) found in the populations of Bangka Island, Bogor, Malang, Pati, and Lombok. The same thing is also shown from the results of nucleotide diversity analysis (π) where the highest value was 0.002358 in the Lampung region and the lowest was 0.0000, namely in the Bangka, Bogor, Malang, Pati and Lombok regions (Table 3).

Genetic distance

The percentage of genetic distance between *P. manihoti* populations was 0.00000-0.00409. The largest genetic distance of 0.00409 between populations occurred in the population of Bangka-Semarang, Cilegon-Semarang, Malang-Semarang, Pati-Semarang, Lombok-Semarang, while the smallest genetic distance between populations occurred between the population of Bangka-Cilegon, Bangka-Malang, Bangka-Pati, Bangka-Lombok, Cilegon-Malang, Cilegon-Pati, Cilegon-Lombok, Malang-Pati, Malang-Lombok, Pati-Lombok, each was 0.0000, while the average genetic distance between populations was 0.000127. The genetic distance between *P. manihoti* from China with samples was 0.00614 - 0.00204. The genetic distance between *P. manihoti* from the whole population with *P. madeirensis* was around 0.14645 (14.645%)-15135 (15.135%) and *P. parvus* was around 0.03357 (3.35%)-0.14108 (14.108%) (Tabel 4).

Based on the *Fst* results through the AMOVA test, the structure of population *P. manihoti* has a variation value between populations found in the entire observation population of 91.96%, while value of intrapopulation variation is 8.04%. The *Fst* value for the entire population was 0.91958 (p = 0.0000).

Table 1. *Phenacoccus manihoti* nucleotide composition

Characteristics	Population							Average	
	Bangka	Lampung	Bogor	Cilegon	Semarang	Malang	Pati		Lombok
Timin (T)	47.1	47.2	47.2	47	47.01	47.1	47.1	47.01	47.09
Sitosin (C)	13.71	13.56	13.56	13.61	13.61	13.71	13.71	13.61	13.64
Adenosin (A)	32.63	32.48	32.48	32.63	32.82	32.63	32.63	32.63	32.61
Guanin (G)	6.56	6.76	6.76	6.76	6.56	6.56	6.56	6.76	6.66

Table 2 Analysis of homology of the mitochondrial CO1 gene

<i>Phenacoccus manihoti</i> individual	The nucleotide site							Haplo type
	1	1	0	1	1	1	1	
Bangka-1, Bangka-2, Bangka-3	A	C	T	C	T	G	A	1
Bangka-4, Bangka-5, Malang-1	
Malang-1, Malang-2, Malang-3	
Malang-4, Pati-1, Pati-2, Pati-3	
Pati-4	
Lampung-1, Lampung-3	T	.	.	T	G	.	.	2
Lampung-2	T	.	.	.	G	.	.	3
Lampung-4	T	A	.	.	G	.	.	4
Bogor-1, Bogor-2, Bogor-3, Bogor-4, Bogor-5	.	A	G	5
Cilegon-1, Cilegon2	.	.	.	T	G	.	.	6
Cilegon-3, Cilegon-4	G	.	.	7
Semarang-1, Semarang-2, Semarang-3	T	A	.	.	G	A	.	8
Semarang-6, Semarang-6	
China	.	.	A	

Table 3. Diversity of *Phenacoccus manihoti* haplotypes and nucleotides at the observation site

Population	n	S	h	Hd	K	π
Bangka	5	0	1	0.00000	0.00000	0.00000
Lampung	4	2	3	0.83333	1.16667	0.002358
Bogor	5	0	1	0.00000	0.00000	0.00000
Cilegon	4	1	2	0.66667	0.66667	0.00000
Semarang	5	0	1	0.00000	0.00000	0.00000
Malang	4	0	1	0.00000	0.00000	0.00000
Pati	4	0	1	0.00000	0.00000	0.00000
Lombok	5	0	1	0.00000	0.00000	0.00000

Note: Number of samples (n), number of polymorphic sites (S), number of haplotypes (h), diversity of haplotypes (Hd) and nucleotide diversity (π), the average number of different (K)

Phylogenetic analysis

Analysis of *kimura-2 parameter* showed that the *P. manihoti* populations were divided into 2 clusters. Therefore, this study successfully proved that there was a clearly interspecific separation based on the construction of the phylogenetic tree (Figure 2). The outgroup sequence

data used in this study are *P. parvus* and *P. madeirensis*, which is genetically the closest relative to *P. manihoti*. The phylogenetic reconstruction describes separate outgroups with all population samples *P. manihoti*.

Discussion

Morphological identification of imago *P. manihoti* is pink, oval in shape, soft in shape, has an antenna, body stretch is obvious and is covered with colored waxy flour, eyes are relatively prominent and the limbs develop well and are the same size. The results of making slide preparations in accordance with the key that is the body have 18 pairs of rows, antennas that have 9 segments, there are denticles on the nails, a circular shaped horn. The length of this pest is around 1.77mm and 0.99mm wide. Quinquelocular pores are found around the anterior head to the *clypeolabral* shield, reaching 32-68. Morphological observations are in accordance with the identification keys of Williams and de Willink (1992). Knowledge of this morphology is needed for the first step towards the success of research and biological control processes. According to Gardner et al. (1991) say one way to find out genetic diversity is to study phenotype differences. Morphological characters are phenotypes of external forms or real characters possessed by an individual.

This study was the first to report molecular identification of *P. manihoti* in Indonesia based on mtDNA COI genes. Understanding the characteristics of COI sequence provides better knowledge into the population's genetic structure (Liu et al. 2013). The variation in mitochondrial sequence is inherited in maternal genomes that are largely derived from gene mutations. COI is one of the mitochondrial coding and variable genes, useful in providing valuable information for the study of intraspecific polymorphisms (Barbaresi et al. 2003). Firstly found in 2010, *P. manihoti* has now spread in several regions in Indonesia. *P. manihoti* samples were found in eight locations, namely Lampung, Bangka Island, Cilegon, Bogor, Pati, Semarang, Malang, and Lombok. Characterization of base sequence of COI gene of *P. manihoti* showed a 518 bp length of the gene. The average nucleotide composition of A + T was higher (79.7%) than G + C (20.3%) in the COI gene; Thymine (46.9%), Adenine (32.8%) while Cytosine (13.6%) and Guanine (6.6%).

Table 4. Genetic distance between and intra *Phenacoccus manihoti* populations

Population	Bangka	Lampung	Bogor	Cilegon	Malang	Pati	Lombok	Semarang	China	<i>P.madeirensis</i>	<i>P. parvus</i>
Bangka	0.00000										
Lampung	0.00255	0.00102									
Bogor	0.00204	0.00357	0.00000								
Cilegon	0.00000	0.00255	0.00204	0.00000							
Malang	0.00000	0.00255	0.00204	0.00000	0.00000						
Pati	0.00000	0.00255	0.00204	0.00000	0.00000	0.00000					
Lombok	0.00000	0.00255	0.00204	0.00000	0.00000	0.00000	0.00000				
Semarang	0.00409	0.00153	0.00204	0.00409	0.00409	0.00409	0.00409	0.00000			
China	0.00204	0.0046	0.00409	0.00204	0.00204	0.00204	0.00204	0.00614	0.00000		
<i>P.madeirensis</i>	0.14645	0.14951	0.1489	0.14645	0.14645	0.14645	0.14645	0.15135	0.14890	0.00000	
<i>P. parvus</i>	0.03568	0.03834	0.0378	0.03568	0.03568	0.03568	0.03568	0.03993	0.03357	0.14108	0.00000

This nucleotide composition value is close to that of *P. manihoti* in Thailand (Rattanawanee and Chongrattanameteekul 2015) who found thymine nucleotide composition of 47.7%, cytosine of 13.7%, followed by adenine of 33.1% and Guanine of 5.5%. The composition of A + T in the mitochondrial gene of insects has a tendency to have a higher number of compositions compared to the base content of G + C (Simon et al. 1994). One characteristic of mitochondrial DNA in insects is that it has a high A + T composition (Liu and Beckenbach 1992; Simon et al. 1994). The higher composition of weak hydrogen A + T bonds causes this gene to quickly changes as compared to other genes with higher percentage of the stronger hydrogen bonds between G + C nucleotides.

Multiple alignments of COI genes from eight populations with total number of 36 individuals showed that there are 434 base pairs (83,622%) of the total length of 518 base pairs nucleotides which have similarities to *P. manihoti*. Hoy (1994) mentioned some cases of thelytoky, where the egg only develops after penetration by sperm (pseudogamy or gynogenesis), but the sperm nucleus degenerates without joining the nucleus of the egg, so it provides no genetic contribution to the embryo. Then, the COI gene has many advantages for studying genetic characteristics because it has very little deletion and insertion in its sequence, and many parts of this gene are conserved so that it can be used as DNA barcoding in most species (Hebert et al. 2003). Besides the arrangement of amino acids from proteins encoded in the COI, this gene rarely undergoes substitution, so that the COI gene is stable and can be used as a marker in phylogenetic analysis, but silent mutations are still occurring on the third bases of its codons are (Lynch and Jarrell 1993).

Genetic distance between populations is important information for studying DNA transfer from one population to another so that it can influence the micro-evolution of a population or species (gene flow). Freeland (2005), the factors that influence gene flow in a population are migration, limiting factors for migration, reproduction, habitat fragmentation, and metapopulation, and interspecies interactions. Based on the knowledge of genetic flow

between populations through Fstatistik values, it will be known the occurrence of fragmentation or differentiation in a population. Based on the results of the FST through the AMOVA test, the population structure of *P. manihoti* has a value of the interpopulation variation of 91.96%, while the value of the intrapopulation variation of 8.04%. While the overall Fst value is 0.91958 ($p = 0.0000$). The result of genetic differentiation between populations of *P. manihoti* shows that there is a genetic structure between populations. The low gene flow between populations is possible because *P. manihoti* is thelytoky parthenogenesis.

Specific nucleotide sites were found at the 12th position with nucleotide T (Timin) characteristics for Indonesia and A (Adenine) for China. This shows the difference in population of *P. manihoti* from Indonesia with that from China (gene Bank data). Likewise, Rattanawanee and Chongrattanameteekul (2015) stated that the results of *P. manihoti* research in Thailand obtained DNA fragments containing 419 base pairs (bp) from the COI gene. This result is different from the base length results carried out in this study amounting to 518 bp. Li (1997) stated that different nucleotide variations in the new haplotype are the result of changes in transitional and transversal substitution types. The haplotype found in this study, from all populations, had differences with the haplotypes in GenBank, so it can be concluded that the Indonesian haplotype is a new haplotype.

The results of the phylogeny tree analysis using *kimura-2 parameter* show that the population of *P. manihoti* is divided into 2 clusters, but still shows a low bootstrap value. This indicates that *P. manihoti* in Indonesia has a very close relationship. Outgroup sequence data used in this study were *P. parvus* and *P. madeirensis*, which were genetically the closest relatives of *P. manihoti* species. The results of phylogenetic reconstruction describe separate outgroups with all *P. manihoti* population samples. This study also succeeded in showing that it was successful in proving that there was a clear interspecies separation based on phylogenetic tree construction, and also the relationship between morphological and molecular characterizations that supported each other. Research result of

Rattanawanee dan Chongrattanameteekul (2015) who used mtCO1 DNA to identify *P. manihoti* obtained phylogenetic results into 2 clusters. Far phylogenetic differences between populations indicate significant differences in population structure (Zhao et al. 2013).

Phylogenetic trees can also be a reference to relationship based on geographical location, so that they can indicate the relationship between individuals and species through phylogenetic reconstruction (Vélez-Zuazo and Agnarsson 2011).

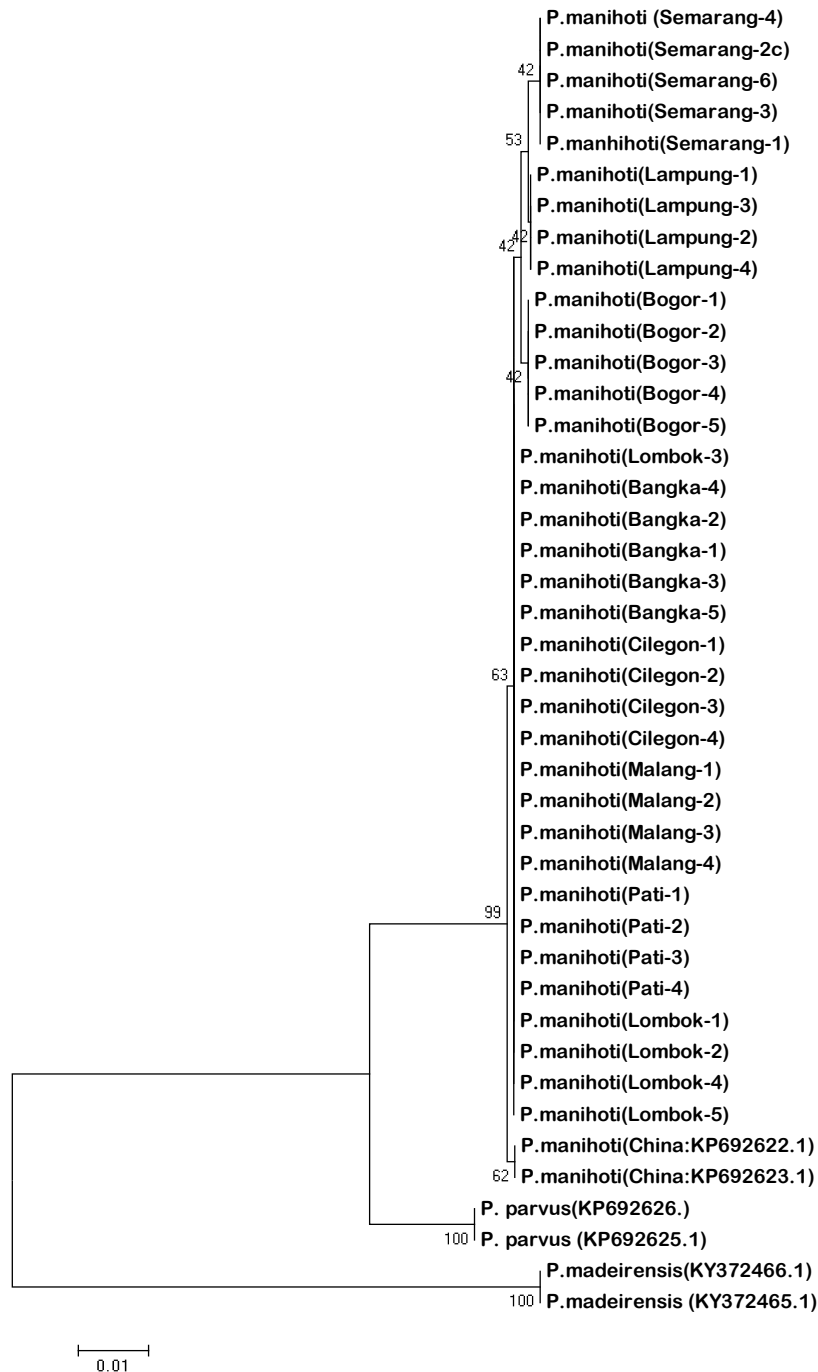


Figure 2. Phylogenetic reconstruction *Phenacoccus manihoti* from 8 observation populations in Indonesia using the method of Neighbor-Joining (NJ) with Kimura 2-parameter model

Phylogenetic reconstruction of eight populations of *P. manihoti* using the Neighbor-Joining method. Outgroup sequence data used in this study are *P. maderensis* and *P. parvus* species which are genetically the closest relatives of *P. manihoti* species. Other sequential data from China were also downloaded from GenBank to be used as a comparison of data obtained from eight *P. manihoti* populations found in Indonesia. Data from the phylogenetic reconstruction represent two clusters. The first cluster consisted of all *P. manihoti* populations in Indonesia with bootstrap 42 while the second cluster of *P. manihoti* originating from China (GeneBank data) with bootstrap 63. Whereas between *P. manihoti* populations with outgroup bootstrap values were 99. Based on two phylogenetic clusters *P. manihoti* formed, found one group with the most dominant population compared to other groups. The group consists of the population of Lombok, Bangka, Cilegon, Malang, Pati, Lombok. This is also supported by the low value of genetic distance and the presence of genetic structure between populations so that there is a suspected gene flow between populations, so the potential for phylogenetic kinship to form the same group tends to occur (Zhao 2013). The large number of populations that occur in this cluster according to Kosztarab and Kozár (1988), it is possible to spread helped by the wind, water, land, humans, and animals and wild animals. As well as their vague habits and their small size so that white lice are difficult to detect, especially if their population density in plants is low (Gullan and Martin 2003).

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