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Genotyping of Tropical Bed Bugs, *Cimex hemipterus F.* from Selected Urban Area in Malaysia Inferred from Microsateelite Marker (Hemiptera: Cimicidae)

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Abstract

A total of 9 populations of tropical bed bug (*Cimex hemipterus*) collected from urabn area in Penang and other locations in Malaysia. The populations involved in this study included Sungai Ara, Taman Brown, Desa Permai Indah, Tasek Gelugor and Lahar Tembun in Penang, Shah Alam, Petaling Jaya, Pahang and Seremban, Malaysia. Deoxyribonucleic Acid (DNA) was extracted from total 5 individual bed bugs each location and used as a template for the Polymerase Chain Reaction (PCR) to amplify 5 different genomic microsatellite loci. PCR products were resolved by agarose gel electrophoresis and visualized with ethidium bromide staining. The genotyping data from the sample sites revealed that PCR-based genotyping reliably separated the samples into genotypic groups corresponded to the location.

Keywords: Tropical bed bugs; *Cimex hemipterus;* Microsatellite marker; Genotyping

Introduction

Bed bugs are insect which small in size, lack of wing, obligate hematophagous ectoparasite and have lived in close association with humans [1]. There are two common types of bed bug: *Cimex lectularius* and *Cimex hemipterus*. They feed mainly on human blood; however, they can feed on a wide range of hosts such as bats, birds and infrequently various domestic animals. Bed bugs are mostly found in public places such as apartments, hotels, public transport system, health care facilities and residences areas, which they usually hide in sleeping areas, furniture, clothing and others [1].

Cimex hemipterus is the common type of bed bugs in Malaysia because it usually can live in the tropical areas. Recently, the cases of bed bug infestations are rising at a fast rate in urban center globally [2]. Therefore it is necessary has a research on bed bugs biology because the causes of resurgence are not clear due to reluctance knowledge or information about the geographical sources of the resurgent populations and their patterns of spreading [3]. Some possible reasons for infestation of bed bugs include greater international commerce and travel, host switching, changes in pest control practices and insecticide resistance [4]. Bed bugs infestations were common in hostels, public accommodations and resident premises.

In recent year, genetic variation at the molecular level (protein and DNA) has been generally used to study the genetic diversity and genetic relationship among individuals and populations. Molecular markers are mostly used for study and analysis of genetic biodiversity which are based on DNA sequence polymorphisms. In fact, diversity of organisms can be determined by DNA sequences and hence, the methods used to estimate DNA polymorphisms directly determine the genetic diversity.

Microsatellite markers are common molecular markers used to analyze genetic diversity. Microsatellites are tandem repeats of 1-6 nucleotides [5] that can found in the genome of prokaryotes and eukaryotes. Microsatellites exhibit high level of polymorphism [6] and mutation rate [7] so microsatellites are variable and can be a good molecular marker for analysis genetic diversity. In this study, genetic analysis of several populations of bed bugs, *C. hemipterus* in Penang and several other locations in Malaysia was genotyped using microsatellite marker.

Materials and Methods

Sample collection

In this study, bed bugs were collected from various locations in the Penang and other locations in Malaysia. Bed bug samples were collected by using forceps then stored in a sample collection bottle. Bed bugs were preserved in 95% alcohol and stored at -20°C (Table 1). A total of 45 bed bug samples were used which each population has 5 bed bug samples.

DNA extraction

The bed bug DNA was extracted by using the QIAamp DNA Mini Kit (Qiagen) [8]. The 5 bed bug samples from each location were

State	Location	Latitude/Longitude
Penang	Taman Brown	5°22' N/100°17' E
	Lahar Tembun	5°28' N/100°28' E
	Tasek Gelugor	5°29' N/100°30' E
	Sungai Ara	5°19' N/100°16' E
	Desa Permai Indah	5°20' N/100°17' E
Other locations in Malaysia	Seremban	2°43' N/101°56' E
	Shah Alam	3°5' N/100°30' E
	Petaling Jaya	3°6' N/101°36' E
	Pahang	3°58' N/102°26' E

Table 1: Source of bedbug samples.

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grinded by pestle and places into 1.5 ml micro centrifuge tube. Then, 180 µl of Buffer ATL was added and followed by 20 µl Proteinase K. The mixture was mixed evenly by vortexing, and incubated at 56°C until the tissue was completely lysed. The 1.5 ml micro centrifuge tube was briefly centrifuged to remove drops from the inside of the lid. After that, 200 µl of Buffer AL was added to the sample, mixed by vortexing for 15 second, and incubated at 70°C for 10 minutes.

Next, 200 µl 96-100% ethanol was added to the sample, and mixed by vortexing for 15 second. The mixture in the microcentrifuge tube was carefully poured into the QIAamp Spin Column (in a 2 ml collection tube) without wetting the rim. The cap was closed, and centrifuged at 6000 x g (8000 rpm) for 1 min. The QIAamp Spin Column was placed in a clean collection tube and discarded the tube containing the filtrate. Then, the QIAamp Spin Column was opened and 500 µl Buffer AW1 was added without wetting the rim. The cap was closed and centrifuged at 6000 x g (8000 rpm) for 1 min. The QIAamp Spin Column was placed in another clean collection tube and also discarded the collection tube containing the filtrate. Subsequently, the QIAamp Spin Column was carefully opened and 500 µl Buffer AW2 was added without wetting the rim. The cap was closed and centrifuged at 20,000 x g (14,000 rpm) for 3 min.

For the elution step, the QIAamp Spin Column was placed in a clean 1.5 ml microcentrifuged tube and discarded the collection tube containing the filtrate. The QIAamp Spin Column was opened carefully and 200 µl Buffer AE was added. Then incubated at room temperature for 1 min and centrifuged at 6000 x g (8000 rpm) for 1 min. Finally the elution step was repeated two times to increase the final DNA concentration.

Microsatellite marker

The five PCR primers used in the genotyping research for bed bug were those developed by Booth et al. [8] who designated the corresponding PCR products for each locus as: 1) BB6B, 2) BB29B, 3) Clec6, 4) Clec21, and 5) Clec43 (Table 2). These five primers set were purchased from NKH Bioscience Solutions Sdn. Bhd. The microsatellite loci were amplified by using Polymerase Chain Reaction (PCR).

Amplification microsatellite loci by PCR

The microsatellite sequences for each locus were amplified through PCR. The PCR was conducted in 25 µl total volume, each containing 5 x PCR buffer, 25 mM $MgCl_2$, dNTPs, DNA template, forward and reverse primers, i-Taq DNA polymerase (iNtRON BIOTECHNOLOGY), and sdH_2O. PCR was carried out using a 'touchdown' program. Touchdown PCR is a useful method by using a cycling program with varying annealing temperatures to increase the specificity of PCR. Thermal Cycler (BIO-RAD) (Plate 3.4) was used for thermal cycling as follows: 1 cycle of 4 min at 94°C; a touchdown protocol composed of 30 cycles of 30 s at 94°C, hybridization for 30 s at 60°C (for the first cycle) decreasing 0.5°C per cycle to 45.5°C in the last cycle, and 1 min at 72°C for extension; 30 cycles of 30 s at 94°C for denaturation, 30 s at 45°C for hybridization, 1 min at 72°C for extension; and 1 cycle of 10 min at 72°C for final extension.

Locus	Primer sequences (5'-3')	Allele size range (bp)
BB6B	Forward: TCTGAAATTTGTAATTGCCTCAA	137-163
	Reverse: CCCCCGTGGGTTTATTATTT	
BB29B	Forward: GCTTCGCCTAACACAAGGA	178-268
	Reverse: CACCTGTACATCTAAAACAGGATACC	
Clec6	Forward: GATACCTGCAACAACC	306-310
	Reverse: CGTGTCACCCTTGGCTTTC	
Clec21	Forward: CCATTCCAACCCTGCTTACTG	278-284
	Reverse: ACGACTAAACATGTCCAAACTC	
Clec43	Forward: AGAGATACCCATTTACCTCGGTG	437-443
	Reverse: GGCAGAAACAAGCCTGTCC	

Table 2: List of primers used for amplification.

PCR purification

The PCR samples were purified or cleaned up by using QIAquick PCR Purification Kit (Qiagen) [9]. The Buffer PB and PCR sample were added in the volume of ratio 5:1 into the QIAquick spin column. The QIAquick spin column was centrifuged at 13, 000 rpm for 1 minute and then the flow-through was discarded and the QIAquick column was placed back into same collection tube. Then, 0.75 ml Buffer PE was added into the QIAquick column and centrifuged at 13,000 for 1 minute. The flow-through was discarded and the QIAquick column was placed back into the same tube and the column was centrifuged for an additional 1 minute. The QIAquick column was placed in a clean 1.5 ml microcentrifuge tube and 30 µl Buffer EB was added into the QIAquick column. The QIAquick column was incubated at room temperature for 1 minute and centrifuged at 13,000 for 1 minute. The DNA samples were kept in the freezer and amplified again by using PCR reaction.

Gel electrophoresis, staining and visualization

The 0.8% agarose gel was prepared first by weighing 0.4 g of agarose powder and then mixed with 50 ml 0.5X TAE buffer in a conical flask. After the agarose gel has solidified, sample loading and electrophoresis could be initiated. The gel mold tray was placed into the running apparatus and 0.5X TAE buffer was added into the chamber until the gel was just submerged. After this, 2 µl PCR products for each DNA samples were pipetted carefully and loaded into the wells of gel. 2 µl of 1k bp DNA ladder (Bio Basic Inc. USA) was mixed with 1 µl 6 X loading dye on the parafilm and then loaded into one lane of gel. The gel was run for 30 minutes at 65 V. After finishing running gel, the gel was placed into a basin which contains ethidium bromide (EtBr) solution and stained for 10 minutes. Then, the gel was transferred into another basin which contains dH_2O to destain the gel for 5-10 minutes. Next, the gel was placed on a UV transilluminator for nucleic acid visualization and the gel was also visualized and photographed directly on a UVP Gel Doc System [10].

Data analysis

The size of allele at each locus can be determined after gel electrophoresis. Each gel band which represents each allele will be scored to determine its size. Scoring was carried out by comparing the alleles with the DNA marker, 1k bp DNA ladder. The data was collected and analyzed.

Results

Microsatellite Loci Amplification: The process of amplification for each DNA microsatellite locus was carried out using PCR to amplify the DNA samples from each population. The PCR products were separated through gel electrophoresis by using 0.8% agarose gel in order to segregate the DNA fragments according to size. The 1k bp DNA ladder (Bio Basic Inc.) was used as marker to determine the size of each DNA fragments.

Table 3 showed the detection of alleles for five loci in nine populations. The entire detected locus and genotyped for each populations were summarize in Table 4. For locus BB6B, only one

allele was detected in five populations (PI, LT, PJ, SH and TB) while the other four populations showed a negative result (Figure 1). A total of two alleles were detected in locus BB29B in which Seremban (S) is the only one population showed two alleles and four populations (PJ, SA, PH and TG) failed to show any band (Figure 2). Locus Clec6 is the locus which showed the most alleles detected that a total five alleles were detected in this locus (Figure 3). The populations from Desa Permai Indah (PI), Lahar Tembun (LT) and Shah Alam (SH) have a total five, three and two alleles detected respectively. The populations from Seremban (S), Sungai Ara (SA) and Pahang (PH) have only one and similar allele detected (Figure 3). Among nine populations, six populations have shown positive result which bands were formed in the locus Clec21. Two populations (PI and SA) have two alleles detected whereas the other four populations (LT, PJ, TB and PH) have only one allele detected (Figure 4). In total nine populations in the locus Clec43, three populations (LT, PJ and SH) have two alleles detected, three populations (S, SA and PH) have only one allele detected and another three populations (PI, TB and TG) failed to show any band (Figure 5).

Discussion

This study is the first research about the genetic genotyping of *C. hemipterus* in Penang and other locations in Malaysia by using high polymorphism microsatellite DNA markers. The AquaGenomic Tissue Kit was used to extract the bed bugs DNA at the beginning of research. However, the OD of extracted DNA samples mostly in the range of 0.5 – 1.8 and the DNA samples which amplified by PCR have shown some bands after gel electrophoresis but the bands showed were unable to score. Therefore, some modifications have performed such as changing the percentage of agarose gel, increasing the DNA concentration by using more than one bed bug sample in DNA extraction and changing the DNA ladder.

Five individuals bed bug were used for each extraction in order to increase the concentration of DNA samples. Gupta and Preet [11] showed the genomic DNA of mosquito larvae, was extracted from single, five and ten samples separately has high DNA yield and absorbance in DNA samples contain more mosquito larvae. Finally, nine DNA samples from nine different populations were extracted and the OD of DNA samples in the range of 1.1 – 1.9. The extracted DNA samples were stored at -20°C to prevent the DNA samples have been contaminated or denatured. The DNA samples must be having a long-term storage such as by frozen which allow the DNA samples suitable for use in subsequent procedures and reanalysis [11].

The selected DNA microsatellite loci were amplified through PCR. All PCR process must be carried out aseptically to prevent contaminated by bacteria. Therefore, the mixing of PCR components must perform in the laminar flow hood to reduce potential cross-contamination [12]. The materials used such as pipette, pipette tips, eppendorf tube and PCR tube must be sterilized and autoclaved. The PCRs were carried out in 15 µl total volumes and the PCR cycling conditions were referred to [1] at the beginning of research. However, the desirable results were not obtained so a 'touchdown' program PCR were chose to amplify the microsatellite loci and PCRs were carried out in 25 µl total volumes. Touchdown PCR was chosen because it can optimize PCR more rapidly and increasing specificity, sensitivity and yield [13].

The PCR products will load into the 0.8% agarose gel to separate

Locus	Populations								
	PI	LT	PJ	SH	TB	S	SA	PH	TG
BB6B	√	√	√	√	√	X	X	X	X
BB29B	√	√	X	√	√	√	X	X	X
Clec6	√	√	X	√	X	√	√	√	X
Clec21	√	√	√	X	√	X	√	√	√
Clec43	X	√	√	√	X	√	√	√	X

√: Allele detected; X: No allele detected

Table 3: The detection of alleles for five loci in nine populations.

Locus	Population	N	No. of Alleles	Genotype
BB6B	PI	5	1	AA
	LT	5	1	AA
	PJ	5	1	AA
	SH	5	1	AA
	TB	5	1	AA
	S	1	0	
	SA	1	0	
	PH	1	0	
	TG	1	0	
			Total = 1	
BB29B	PI	5	1	AA
	LT	5	1	AA
	PJ	5	0	
	SH	5	1	AA
	TB	5	1	AA
	S	1	2	AB
	SA	1	0	
	PH	1	0	
	TG	1	0	
			Total = 1	
Clec6	PI	5	5	AB, CD, EE
	LT	5	3	AA, CD
	PJ	5	0	
	SH	5	2	AB
	TB	5	0	
	S	1	1	BB
	SA	1	1	BB
	PH	1	1	BB
	TG	1	0	
			Total = 4	
Clec21	PI	5	2	AB
	LT	5	1	AA
	PJ	5	1	AA
	SH	5	0	
	TB	5	1	AA
	S	1	0	
	SA	1	2	AB
	PH	1	1	AA
	TG	1	0	
			Total = 2	
Clec43	PI	5	0	
	LT	5	2	EF
	PJ	5	2	EF
	SH	5	2	EF
	TB	5	0	
	S	1	1	FF
	SA	1	1	EE
	PH	1	1	EE
	TG	1	0	
			Total = 2	

N: The number of individual bed bug used in one extraction

Table 4: Microsatellite genotypes of *Cimex hemipterus* from five collection sites.

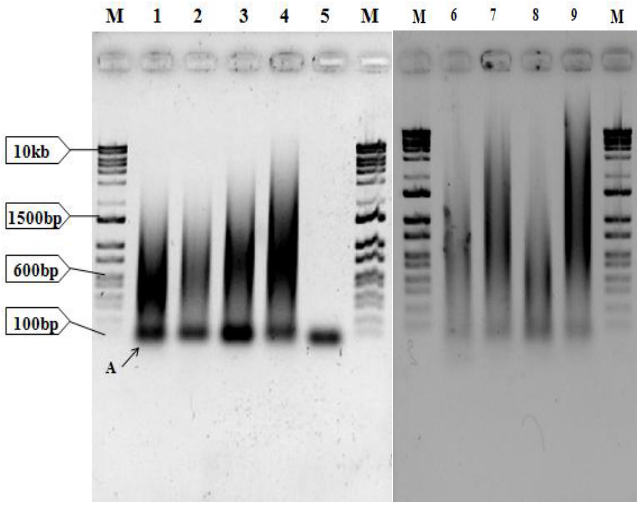

Figure 1: Microsatellite genotypic data of *Cimex hemipterus* for (Locus: BB6B) from samples PI (lane 1), LT (lane 2), PJ (lane 3), SH (lane 4), TB (lane 5), S (lane 6), SA (lane 7), PH (lane 8) and TG (lane 9) using the PCR primer BB6B. PCR products were electrophoresed on a 0.8% agarose gel and stained with ethidium bromide. Alleles sized against 1k bp DNA ladder (lanes M). (A: 100 bp).

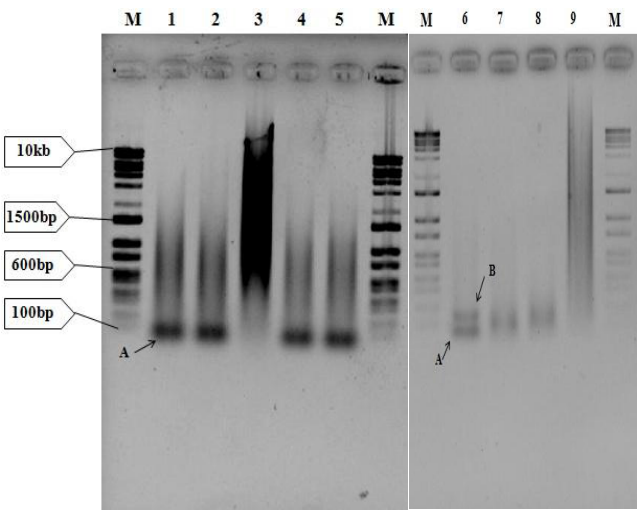

Figure 2: Microsatellite genotypic data of *Cimex hemipterus* (Locus: BB29B) from samples PI (lane 1), LT (lane 2), PJ (lane 3), SH (lane 4), TB (lane 5), S (lane 6), SA (lane 7), PH (lane 8) and TG (lane 9) using the PCR primer BB29B. PCR products were electrophoresed on a 0.8% agarose gel and stained with ethidium bromide. Alleles sized against 1k bp DNA ladder (lanes M). (A: 100 bp; B: 150 bp).

the DNA fragments. The 0.8% agarose gel is used after comparing with different percentages of agarose gel included 1.0%, 1.7% and 2.0% and the results indicate that 0.8% agarose gel was the best one to show clear bands on the gel. The percentage of agarose gel used is dependent on the size of DNA fragment [14,15]. The smaller DNA fragments must require high percentage gel because small pore size has better resolution while large DNA fragments can be resolve on 1% gel. The bands on the gel sometime stick together which did not separated well due to the gel was stained too longer in the ethidium bromide solution. Otherwise, the bands will not look so clear when stained in short time which will make the scoring job more difficult. Therefore, the gel must be stained about 10 min depends on the concentration of ethidium bromide solution.

In this study, the levels of genetic diversity for 9 populations of *C. hemipterus* collected from 5 locations in Penang and 4 locations in Malaysia were analyzed by using 5 microsatellite loci. Across all populations, a relatively low genetic diversity was observed with 1 – 5 alleles per locus (average 2.4 per locus). This allelic diversity was relatively low when compared to the study by Saenz et al. [2] which studied the genetic diversity of *C. lectularius* in United States with the results have 5 – 17 alleles per locus. The low levels of allelic diversity for populations of *C. hemipterus* may due to the small population size used

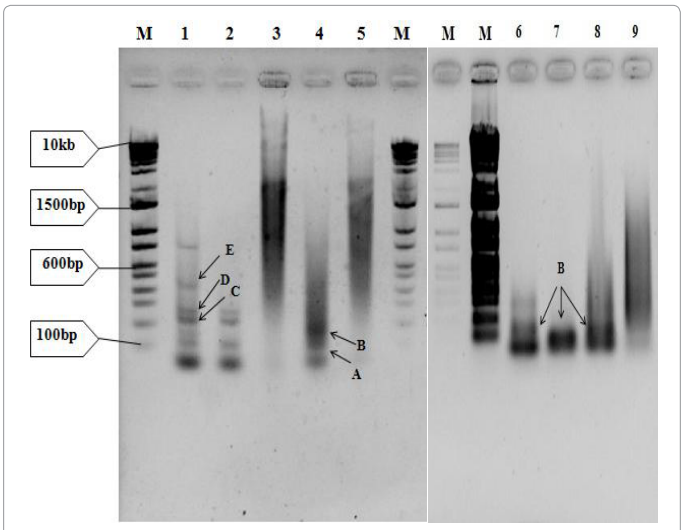

Figure 3: Microsatellite genotypic data of *Cimex hemipterus* from (Locus: Clec6) samples PI (lane 1), LT (lane 2), PJ (lane 3), SH (lane 4), TB (lane 5), S (lane 6), SA (lane 7), PH (lane 8) and TG (lane 9) using the PCR primer Clec6. PCR products were electrophoresed on a 0.8% agarose gel and stained with ethidium bromide. Alleles sized against 1k bp DNA ladder (lanes M). (A: 100 bp; B: 150 bp; C: 200 bp; D: 250 bp; E: 400 bp).

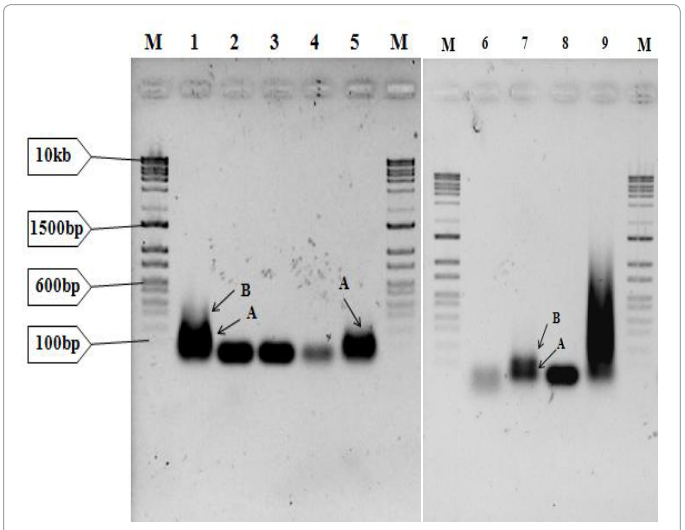

Figure 4: Microsatellite genotypic data of *Cimex hemipterus* from (Locus: Clec21) samples PI (lane 1), LT (lane 2), PJ (lane 3), SH (lane 4), TB (lane 5), S (lane 6), SA (lane 7), PH (lane 8) and TG (lane 9) using the PCR primer Clec21. PCR products were electrophoresed on a 0.8% agarose gel and stained with ethidium bromide. Alleles sized against 1k bp DNA ladder (lanes M). (A: 100 bp; B: 150 bp).

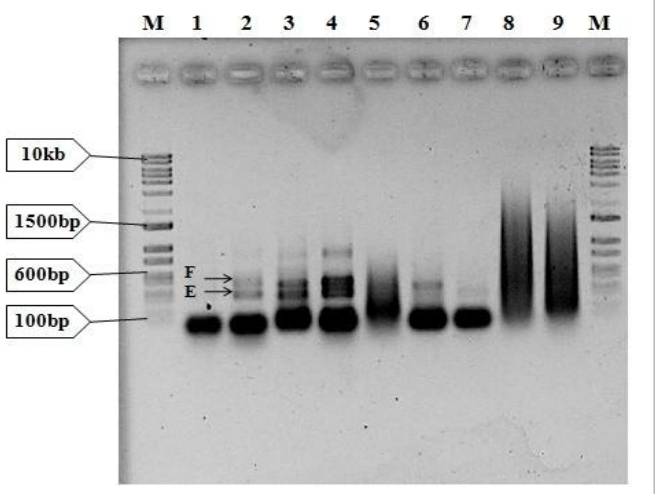

Figure 5: Microsatellite genotypic data of *Cimex hemipterus* from (Locus: Clec43) samples PI (lane 1), LT (lane 2), PJ (lane 3), SH (lane 4), TB (lane 5), S (lane 6), SA (lane 7), PH (lane 8) and TG (lane 9) using the PCR primer Clec43. PCR products were electrophoresed on a 0.8% agarose gel and stained with ethidium bromide. Alleles sized against 1k bp DNA ladder (lanes M). (E: 400 bp; F: 500 bp).

in this research. Unlike a total of 206 individuals at the 9 microsatellite loci were used in the research by Saenz et al. [2], each population only has one replicate in this study due to the limited number of samples available.

The results showed that not all populations will reveal band in each locus. Lahar Tembun is the only one population reveals the bands in all loci while Tasek Gelugor is the only one population did not show any band in all loci. The number of microsatellite loci used in this study was less than the previous literature such as 9 and 25 microsatellite loci were used in the research by Saenz et al. [2] and Vargo et al. [3], respectively. Therefore, more markers are required to study the genetic differentiation for this research which has small population sizes. The presence of null alleles which are mutant copy of a gene could also affect the results that absence of gene product at molecular level. The insects are the one of taxa which has high frequency of null alleles [16].

Conclusions

The results showed that there are 1-5 alleles per locus with a total of 6 alleles are observed in all loci. The allelic diversity is low in this study compared to the Saenz et al. [2] study which showed high allelic diversity with 5-17 alleles per locus. Some suggestions need to be considered to improve this research the sources of samples from more cities or states in Malaysia are better to study the genetic diversity of *C. hemipterus* in Malaysia. Besides that, a bigger the population sizes must be used that about 20-50 individuals per population in order to high allelic diversity results can be observed. The more replicate used, the more easy to compare and analyze the results. The microsatellite markers which are high polymorphism and a good marker used to study the genetic diversity of *C. hemipterus* populations [17,18]. Furthermore, this research was the first preliminary tropical bed bugs genotyping that will help to further investigate infestation dynamic pattern in Malaysia and other tropical region.

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