Microsatellite

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Isolation and Characterization of *Aedes aegypti* Microsatellite Markers Dengue Hemorrhagic Fever Vector in West Sumatra, Indonesia

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ABSTRACT

Aedes aegypti is the major vector of DHF virus in the tropical and subtropical. The DHF prevention depends on vector control because the vaccine is still in development. Microsatellite has become on effective marker to obtain information about genetic diversity and analyze the structure of genetic population. The aim of this study was to isolate and characterize *A. aegypti* microsatellite markers, the Dengue Hemorrhagic Fever (DHF) vector in West Sumatra. Sequences containing microsatellites were obtained by enrichment method. Stages of works were as follows: isolation of *A. aegypti* genomic DNA, restriction with enzymes, ligation with adapters and hybridization by using microsatellite motifs. Furthermore, the candidate fragment contained motifs cloned on plasmid *pGEM T* easy vector using *E. coli* DH 5α with blue-white colony screening. The results showed 46 clones were successfully extracted from a total of 152 clones and became microsatellite motifs with repetition: (GA)3, (CTA)3, (GA)3 (TAAG)3, (ACTT)3 (TC)3 (AC)3. Eight pairs of primer were successfully designed from sequences containing microsatellite motifs with feasible flanking regions. The primer evaluation used 32 DNA samples of *A. aegypti* from 8 cities (population) in West Sumatra. These markers have been successfully amplified 9-17 alleles with amplification products ranging from 129-306 bp, with a high degree of polymorphism. *Aedes aegypti* microsatellite markers obtained can be used to analyze the structure of genetic population of *A. aegypti* and the obtained results were the additional microsatellite markers type of *A. aegypti* than what had previously existed.

Key words: *Aedes aegypti*, genetic markers, microsatellite, primer design, DHF

INTRODUCTION

Aedes aegypti mosquito, the vector of Dengue Hemorrhagic Fever (DHF) disease is a disease that becomes major problem in the tropics and subtropics. The DHF is the most important arbovirus disease in humans (Effler *et al*., 2005; Weaver and Reisen, 2010). This mosquito can also transmit yellow fever virus (Wallis *et al*., 1983) and Chikungunya virus (Van den Hurk *et al*., 2010).

Reducing *A. aegypti* population is the main way to fight against DHF virus because efficient therapy and vaccine for DHF is still in development. According to WHO, prevention of DHF is still simply by controlling the mosquito vector of *A. aegypti*. Mosquito control strategies that have been developed for decades yet provide maximum results, because it has not been referred to the overall vector data such as vector character itself. Thus, it is necessary for a genetic control strategies that require genetic markers such as

microsatellite markers. Genetic control strategies require genetic information in the form of genetic diversity of *A. aegypti*. Genetic diversity includes genetic structure, gene flow and differentiation among and between the population, which can be studied using several markers such as allozyme, RAPD, RFLP and microsatellite DNA (Lovin *et al*., 2009). This is a marker that has a high level of confidence (Jarne and Lagoda, 1996).

Microsatellite is also a genetic marker that is often used to study the mating system and population structure (Steffen *et al*., 1993), linkage, chromosome mapping and population analysis (Silva *et al*., 1999). According to Robinson *et al*. (2004), there are five advantages of using microsatellite markers. First, it is distributed in the entire genome. Second, it is codomint. Third it requires a small amount of DNA. Fourth multiple microsatellite alleles can be detected at a single locus using PCR-based filtering. Fifth, the analysis can be carried out semi-automatic.

Research on genetic diversity that is based on microsatellite DNA of *A. aegypti* has been conducted by Huber *et al*. (1999), microsatellites as a marker in *A. aegypti* genetic studies as DHF vector. Ravel *et al*. (2001, 2002) conducted a preliminary study of the genetics populations of *A. aegypti* in Mexico. Lovin *et al*. (2009) did research on the development and validation polymorphic DNA microsatellite and *A. aegypti* genetics population studies in Haiti. Paupy *et al*. (2010) combined the morphological data and DNA microsatellites to investigate the morphological and genetic variability of *A. aegypti* in Niakhar, Senegal. Then Paupy *et al*. (2012), examined the genetic structure and phylogeography of *A. aegypti* vector of DHF and yellow fever in Bolivia. Rasheed *et al*. (2013) examined the structure population of *A. aegypti* in Pakistan.

West Sumatra is one of endemic areas with high cases of DHF every year. Vector mosquito control strategies undertaken during the course was 3 M (Menutup, Closes; Menguras, Drain and Mengubur, Bury) and breeding place of vector and fogging at the time of the case, result yet significant decrease in DHF cases. Further control needed the data of *A. aegypti* genetic diversity using microsatellite markers. These markers can be used as a basis for the development of DHF vector mosquito control strategy. The purposes of this study were to isolate and characterize *A. aegypti* microsatellite markers of DHF vector in West Sumatra.

MATERIALS AND METHODS

Eggs and larvae of *A. aegypti* were collected from houses in Padang and Solok area in May until October, 2012. Eggs were collected with ovitrap and larvae were taken directly in the bathtub. Both were developed in the laboratory until adult. Genom DNA was extracted from whole body of *Ae. aegypti* using cetyltrimethylammonium bromide (CTAB) protocol describ by Hoelzel (1994) with modification by Anggraini (1998). A microsatellite-enriched library was obtained using protocols adapted from Edwards *et al*. (1996) and Zane *et al*. (2002). Genomic DNA from one individual of *A. aegypti* (Padang and Solok) was digested with *Alu*I, *Rsa*I and *Hind*II enzyme (Vivantis), the cutting results were ligated then with *Mlu*I adaptor (consist of a 21-mer: 5'CTC CTT TTG ACG CGT GGA CTA3' and a phosphorylated 25-mer at the 5' end: A 3' ACA CGA CGA GAA GCA CCT GATp5' TG). Ligation reaction contained 1 μL DNA, 0.5 μL 21-mer adapter, 1 μL 25-mer adapter, 5 μL 5x Rapid ligation buffer, 1 μL of T4 DNA ligase. PCR reactions were performed under the following conditions: 30 sec initial denaturation at 94°C 1x, followed by 35 cycles of 30 sec at 94°C, 1 min at 60°C, 2 min at 72°C and 10 min at 72°C. DNA was enriched with $(AT)_n$ $(GT)_n$ $(GT)_n$ $(AC)_n$ $(AG)_n$ $(GC)_n$ motifs (Slotman *et al*., 2007). Microsatellite-enriched DNA fragments were ligated into *pGEM-T Easy* Vector (Promega, 1999), which were used to transform DH5α *Escherichia coli* competent cells (Promega USA Corporation). Positive clones were selected using the β-galactosidase gene and grown overnight with ampicillin. The sequencing process was performed in Macrogen Seoul, Korea, Result was edited by Geneious program 5.5.7 version. Screening of vector sequence was entry into vecscreen in NCBI website, continued with BLASTN to determine the homologues of obtained sequence and compared with GeneBank data result. Microsatellite motifs were found with Microsatellite Finder (Bikandi, 2006).

Primer pairs were designed with Primer 3 from sequences which flank the microsatellite and were tested in DNA of mosquitoes (Padang and Solok). Evaluation was also tested on the DNA derived from six cities/other populations (Painan, Pariaman, Ujung Gading, Lubuk Sikaping, Bukittingi and Payakumbuh). The PCR was program consisted of 5 min at 94°C followed by 35 cycles of 30 sec at 94°C, 1 min (optimal annealing temperatures; 55-67°C), 2 min at 72°C and 10 min at 72°C. The amplification products were separated under denaturing conditions on a 10% Polyacrylamide gel (PAGE) for 2.5 h at 75 volts using a Mini Protean BIORADs System The microsatellite fragments position was two fragments, it as heterozygous determined by looking at the number of fragments. If there were two fragments were heterozygous and it there was one fragment, it was homozygous. The supporting assumption revealed that all fragments that have the same rate on polyacrylamide gel was the homologous allele (Nei, 1987). Estimation length of alleles was performed using Image J program by converting the distance of marker on each gel.

RESULTS

The fragments of *A. aegypti* genomic DNA extraction were good enough, because the concentrations varied from 3.6-60.9 ηg μL^{-1} and from 1.82-2.52 purity. Restriction using *Alu*I, *Rsa*I and *Hind*II enzyme resulted in 100-1000 bp DNA fragment (Fig. 1). Candidates of microsatellite motif after enrichment obtained good results (Fig. 2). Forty-six clones were successfully extracted from a total of 152 clones and

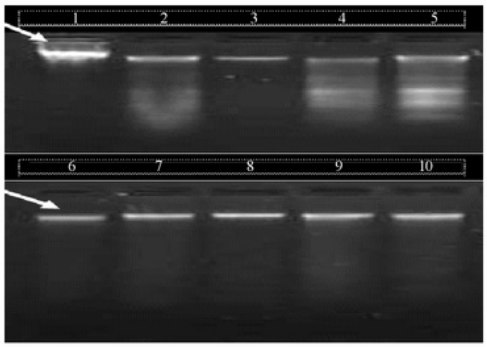

Fig. 1: An example of DNA fragment isolation results of *Aedes aegypti*

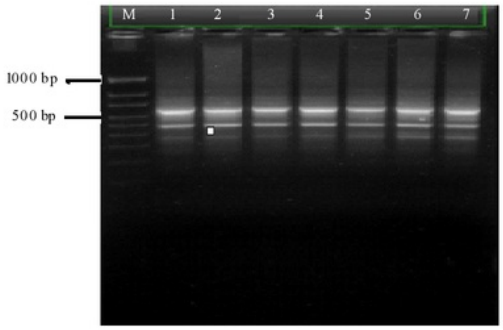

Fig. 2: DNA fragments candidate with microsatellite motif, 500 bp of fragment, 400 bp in the samples 1, 2, 3, 4, 5, 6 and 7

became candidates of microsatellite motifs (Fig. 3 and 4a), verified again by PCR using 21-mer (Fig. 4b). The results of sequencing using T7SP6 primer contained microsatellite motifs (Table 1).

Characterization of microsatellite motifs was selected based on motif repetition di, tri and tetra-nucleotide (GA, AC, CA, CTA, ACTT and TAAG). The result of primer design can be seen in Table 2. From eight pairs of primer that were designed, only six matched with the sequence of *A. aegypti*. The results of PCR using six primer pairs in 32 samples from eight populations of *A. aegypti* are shown in Fig. 5. Nine to seventeen alleles resulted with target sequence, 129-306 bp amplification product, with high polymorphism.

DISCUSSION

Fragment size of 100-1000 bp obtained from the restriction, genomic DNA was selected with high concentration of extracted DNA.The process was done using a three blunt-tipped enzymes simultaneously. It aimed at

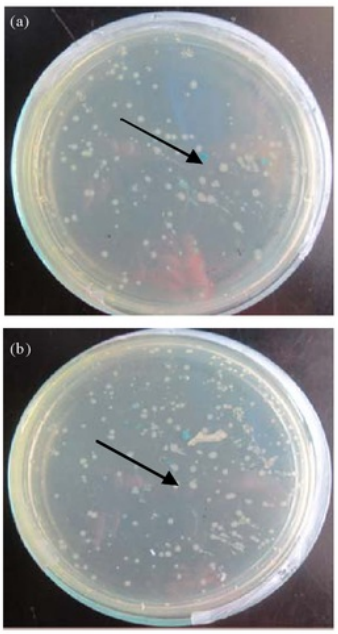

Fig. 3: An example a duplicate of colonies resulted from the transformation, (a) Blue colonies, non-recombinant and (b) White colonies, recombinant

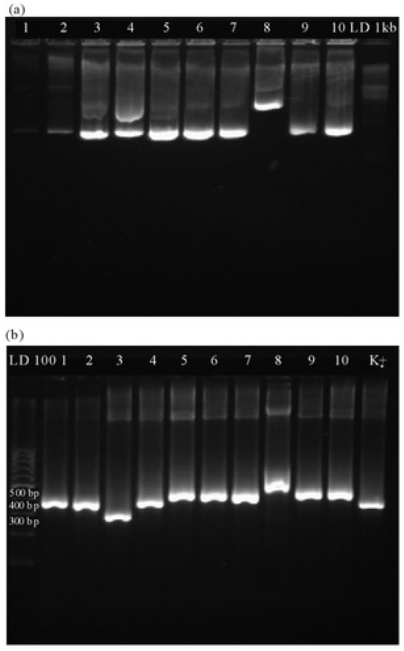

Fig. 4(a-b): (a) Electrophoregram of plasmid DNA from recombinant colony and (b) Electrophoregram of recombinant plasmid DNA, PCR with primers 21-mer, LD: Leader, Samples, 1, 2, 3, 4, 5, 6, 7, 8, 9 and 10 Plasmid

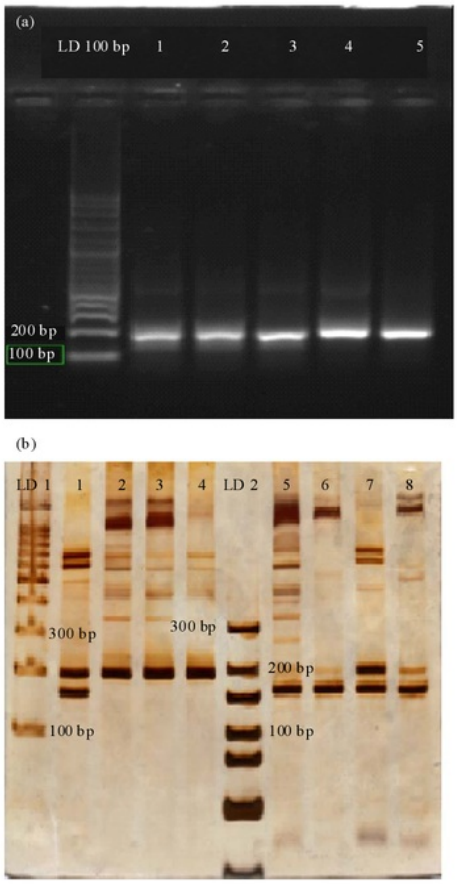

Fig. 5(a-b): (a) Electrophoregram of PCR products by agarose gel and (b) Polyacrylamide gel electrophoresis/PAGE, LD: Laeder 1: 100 bp, 2: 10 bp, samples 1, 2, 3, 4, 5, 6, 7 and 8

improving improve the acquisition of fragment containing the microsatellite motif and facilitate attachment of adapter when ligation (Edwards *et al*., 1996; Kusumawaty *et al*., 2005), increasing the chances of getting microsatellite motifs and reducing the same insert fragments (Zane *et al*., 2002).

The process of enrichment was performed using six types of microsatellite motif; $(AT)_{20}$, $(CT)_{20}$, $(GT)_{20}$, $(AC)_{20}$, $(AG)_{20}$ and $(GC)_{20}$. A total of six microsatellite motifs were grouped into three membrane based on Tm (Edwards *et al*., 1996; Kusumawaty *et al*., 2005), continued with hybridization

Table 1: Microsatellite motif resulted from the analysis of recombinant plasmid

Sample code of DNA	Motif	Length of sequence (bp) of sequencing result
4P35 (1)	$(CTA)_3$	300
4P35 (2)	$(GA)_3$ $(TAAG)_3$	370
4P35 (3)	$(CTTA)_3$ $(TC)_3$	500
4P35 (4)	$(CTA)_3$	300
4P35 (5)	$((GA)_3$ $(TAAG)_3$	420
4P35 (6)	$((GA)_3$ $(TAAG)_3$	420
4P35 (7)	$(TAAG)_3$ $(GA)_3$	400
4P35 (8)	$(GA)_3$ $(TAAG)_3$	420
4S5 (1)	$(CTTA)_3$ $(CT)_3$	450
4S5 (2)	$(GA)_3$	400
4S5 (3)	$(GA)_3$	420
4S5 (4)	$(AG)_3$ $(TAAG)_3$	480
4S5 (5)	$(TACT)_3$ $(TC)_3$	450
4S5 (7)	$(GA)_3$ $(TAAG)_3$	400
4S5 (8)	$((TACT)_3$ $(TC)_3$	450
4S5 (9)	$((GA)_3$ $TAAG)_3$	440
4S5 (10)	$((GA)_3$ $(TAAG)_3$	420
4S28 (1)	$((ACTT)_3$ $(TC)_3$ $(AC)_3$	490
4S28 (2)	$(GA)_3$	360
4S28 (3)	$(GA)_3$ $(TAAG)$	450
4S28 (4)	$(ACTT0_3$ $(TC)_3$	600
4S28 (5)	$((TC)_3$	420
4S28 (6)	$(GA)_3$	450
4S28 (7)	$(GA)_3$ $TAAG)_3$ $(TC)_3$	420
4S28 (8)	$(GA)_3$ (A) 6	420
4S28 (9)	$(GA)_3$ $(TAAG)_3$	440
4S28 (10)	$(GA)_3$ $TAAG)_3$	440

Table 2: Microsatellite Primer resulted from DNA genome characterization of *Aedes aegypti*

Code of primers	Primer sequence (5'→3')	Length of PCR product (bp)	Temperature (°C)	No. of allele
Hs Ae 1	GGCGAACCCAGTATCGTGAAATTTCGCCAATTCCCTCGGT	206-266	60.1 60.0	17
Hs Ae 2	AAGGATGAACCACGAGCTCGATCTTGATAAAGCCTCGC	282-306	60.1 55.0	13
Hs Ae3	GCCTCGCCAACAATATTTCGGTCGGAACAAGAAGGCGT	129-151	57.9 57.6	9
Hs Ae 4	AGCAACTTTTCGCTTTGCCCGCAAGCTCTTTGGGGTTTTCG	184-232	59.9 60.0	16
Hs Ae 5	TTCGGCATTGCTTCCGTTTGACTTGCAAGCTCTTGGGGT	163-211	60.1 60.1	15
Hs Ae6	CGCGTGGACTATACTTTCCTGCCTCGCCAACAATATTTC	181-279	57.9 57.9	16
Hs Ae 7	GCCATGAACCGAGGGAATTGCTCTTGCTTACGCGTGGACTA	-	59.3 60.1	-
Hs Ae 8	GGCGAAATATTGTTGGCGAGGCTCACTAGTGATTCTCTTGCTTACGCGTGG	-	65.6 65.6	-

process. Hybridization used HYBON nylon membrane (Amersham, USA) which already contained non-labeled radioactive microsatellite motif. In order to get strong binding, oligonucleotide (motif) at the membrane was exposed to ultraviolet light (UV). Fixation process was made in the oven, useful for the formation of covalent bonds between DNA and membranes. This is in line the argument proposed with Reed and Mann (1985), that the strong bonding between membrane and DNA could be performed by UV radiation.

The success of getting a candidate fragment containing *A. aegypti* microsatellite motif was a very useful result because *A. aegypti* does not have abundant microsatellites such in plants and animals eukaryotes. Meglecz *et al*. (2007) revealed that *A. aegypti* was characterized by a low abundance of microsatellites in the genome.

Microsatellite motifs found did not show much variation of motifs, with short repetition (three times), because of the frequency and type of microsatellite may occur at a rate which varied. Some researchers reported that motif repetition (GT)n in *A. maculatus* occurred approximately once in every 28 kb (Ongnoparut *et al*., 1996), once in every 26 kb in *A. gambiae* and once in every 10,000 kb in *A. aegypti* (Knudson *et al*., 1996; Chambers *et al*., 2007). Three primers of motif in a nucleotide, one primer of motif in tri-nucleotides and two primers of motif in tetra-nucleotides $((CA)_3$, $(GA)_3$, $(AC)_3$, $(CTA)_3$, $(ACTT)_3$ and $(TAAG)_3)$ designed, obtained from 28 samples which had flanking region. Primer designing performed on closest microsatellite motifs or on short flanking regions caused no product in primer that has been designed. Testing in designed primers of 32 samples DNA from eight populations (Padang, Painan, Pariaman, Ujung Gading, Lubuk Sikaping, Bukittinggi, Solok and Payakumbuh) resulted in PCR products. It concluded this result that the pairs of primer were successfully designed and could be used to analyze the structure of *A. aegypti* population in West Sumatra. Separation of product size for detecting alleles produced homozygous and heterozygous alleles, indicated the presence of polymorphism.

CONCLUSION

There are several conclusions of this study; First, the results of extraction and characterization of the genome of *A. aegypti* by enrichment method through the process of restriction, hybridization and cloning produced some of microsatellite motifs; di-nucleotides, tri-nucleotides and tetra-nucleotides (AC, AG, CTA, ACTT and TAAG). Second, we were able to design successfully eight pairs of primer, six pairs produced good products of amplification, resulted nine to 17 alleles, with 129 -306 bp amplification product. Third, the alleles were polymorphic and could distinguish whether homozygous and heterozygous individuals. Finally, we obtained *A. aegypti* microsatellite markers and could be used to analyze the genetic structure of *A. aegypti* populations and could increase the number of preexist microsatellite markers of *A. aegypti*.

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