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PRIMER NOTE

Isolation and characterization of polymorphic microsatellite markers of *Anopheles sinensis*, a malaria vector mosquito in the East Asia region

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Abstract

Eleven polymorphic dinucleotide (GA and CA) microsatellites were isolated and characterized in the mosquito *Anopheles sinensis*; this species is distributed over the East Asia region and is a primary vector of malaria, particularly in Korea. The number of alleles per locus ranged from 4 to 13. The observed heterozygosity (H_O) and expected heterozygosity (H_E) varied from 0.30 to 0.89 and from 0.59 to 0.90, respectively. These microsatellites could be useful in studying the evolution of the widely distributed *A. sinensis* in diverse environments.

Keywords: *Anopheles sinensis*, East Asia, Korea, malaria vector, microsatellite

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Anopheles sinensis capable of transmitting malaria parasites is widely distributed throughout the East Asia region, extending from the Philippines to Japan. This species is a primary vector mosquito, particularly in Korea, and hundreds of people get infected every year in spite of its zoophilic preference (Chae 1999; Ree 2005). This species has some noteworthy properties in addition to being a malaria vector. Within its distributional range, *A. sinensis* appears to be well-adapted to diverse climatic regions, such as the tropical, subtropical, and temperate regions. In addition, its region of distribution includes a number of isolated locations such as islands and peninsulas where mosquito populations may be geographically separated from those found on the mainland of Asia. Here, we isolated and characterized polymorphic dinucleotide microsatellites of *A. sinensis*.

Genomic DNA was isolated from nonblood-fed individuals that were caught in a light trap by using the standard DNA isolation protocol (Sambrook & Russell 2001). An enriched genomic library for dinucleotide microsatellites was constructed by the method of Hammond *et al*. (1998). Digested DNA fragments sized between 200 and 800 bp were excised from the agarose gel and were recovered by the QIAEX II gel extraction kit (QIAGEN). After SAULA/B linkers (Hammond *et al*. 1998) were attached to the selected fragments by T4 DNA ligase, Polymerase chain reaction (PCR) was performed using linker-ligated fragments as templates. The reaction mixtures (total 25 μL) were composed of 2 μL of linker-ligated DNA, 6.25 μL of SAULA (10 μM) as primer, 1.5 μL of dNTP solution (10 mM), 5 μL of $MgCl_2$ (25 mM), 2.5 μL of *Taq* buffer (10×), 0.5 μL of *Taq* DNA polymerase (Promega), and 7.25 μL of distilled water (DW). PCR amplifications were conducted using GeneAmp 9700. The PCR conditions were as follows: initial denaturation at 72 °C for 5 min, followed by 30 cycles of denaturation at 94 °C for 30 s, annealing at 67 °C for 30 s, elongation at 72 °C for 2 min, and final extension at 72 °C for 7 min. Fragments containing microsatellites recovered by gel extraction method were selected using biotinylated dinucleotide probes (GA_{22} and CA_{22}) and Vectrex Avidin D (Vector Laboratories). The denatured fragments (30 μL) were mixed with 3.5 μL of each biotinylated probe (100 μM), 232 μL of 5 M sodium phosphate buffer (pH 7.4), and 230 μL of DW, and they were then hybridized with the biotinylated probes with gentle shaking overnight at 50 °C. DNA bound with probes was recovered by the avidin matrix and used in subsequent PCRs to amplify the selected fragments under the same conditions mentioned previously. This enrichment process was repeated three times.

After enrichment using the restriction enzyme Sau3AI, SAULA/B, linkers were removed from the enriched DNA that was ligated with a *Bam*HI-digested pUC18 vector (Pharmacia) and were inserted into JM109 competent cells

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Table 1 Characteristics of 11 microsatellite loci in the malaria vector mosquito, *Anopheles sinensis*

Locus Accession no.†	Repeat motif	Primer sequences (5′–3′)	T_a (°C)	No. of alleles	Allele size range (bp)	H_O‡ (H_E)
ANS014 DQ508745	$(GT)_{30}G(GT)_4$	F: FAM-GGTTGGGTTAAAACGCG R: CTGCTGAATGCTCCTC	52	12	125–173	0.35*** (0.83)
ANS035 DQ508746	$(TG)_7AG(TG)$	F: FAM-GAGTCGCATCCATCCGT R: TCTCCCATGTCCAACCC	55	12	110–150	0.65 (0.81)
ANS036 DQ508747	$(CA)_{10}$	F: FAM-ACGTCGAACCAGTCGAAG R: GGCTGGCCTTGCAGCGC	55	7	279–291	0.64 (0.72)
ANS040 DQ508748	$(CA)_2CT(CA)_{11}$	F: FAM-TTCCGTCCCGTTATCCTCG R: CCGGAAAAGTCACAGGG	55	7	209–225	0.70 (0.78)
ANS101 DQ508749	$(GT)_5N_4(GT)_4N_5(GT)_3N_4(GT)_2N_2(GT)_4$	F: FAM-TCCAGATTCCCTACGTACGTG R: ATGGGGCGCTCACACCTCCAA	55	4	211–219	0.52 (0.59)
ANS102 DQ508750	$(GT)_{13}$	F: HEX-ACGACGCTGTGGACGTGCTTA R: TAGGTTTAGAGAGACGCACAC	55	8	230–246	0.70 (0.77)
ANS103 DQ508751	$(CT)_2C(CT)_{12}C$	F: HEX-TCTGTCTGGCCACTTCTGGCC R: CAAGAATGGTGCGCCGTTTGT	55	13	151–209	0.89 (0.90)
ANS107 DQ508752	$(GT)_{10}AT(GT)_2T(GT)_2$	F: HEX-AAGTGCATTTTATCCACCAGG R: CATCGTAAATAATACTCATCC	52	8	195–213	0.59* (0.83)
ANS113 DQ508753	$(GT)_2GC(GT)_{21}GC(GT)_{10}$	F: HEX-TGCGACGCCCAAAAGGACAAG R: CTTTTATTTTGTTTCCTCCTCCGG	55	11	126–306	0.30*** (0.83)
ANS121 DQ508754	$(CA)_{13}$	F: HEX-TCAATTCCGCCACTCAACGTT R: ACGCGTCCACCTTTACCAGGT	55	10	194–216	0.35*** (0.76)
ANS122 DQ508755	$(CA)_{14}$	F: NED-TATGGAAATTGTGTGCAGCTCGC R: CGCTTCACGCCACAAATCCAA	55	10	230–254	0.83 (0.85)

T_a, annealing temperature; H_O, observed heterozygosity; H_E, expected heterozygosity; †GenBank Accession no.; ‡*P*-value of Hardy–Weinberg equilibrium test (*, < 0.01; **, < 0.001; ***, < 0.0001).

(Promega). Correctly transformed clones were picked and replated onto new agar plates that were then used to perform colony hybridization to confirm the presence of microsatellites using DIG-labelled probes and DIG detection kit (Roche). Strongly positive clones were selected and incubated in liquid Luria-Bertani media from which plasmids were recovered using the QIAprep Spin Miniprep Kit (QIAGEN). Inserted fragments were sequenced using the BigDye Terminator Ready Reaction Mix (Applied Biosystems) and M13 primer. Sequences were determined using the Genetic Analyser 3770 (Applied Biosystems) and were finally confirmed visually using SEQUENCE NAVIGATOR 1.1 (PE). PCR primers that were used to amplify the microsatellite region were designed from each clone sequence using the program PRIMER3 (Rozen & Skaletsky 2000). One of the two primers used to amplify each locus was labelled with a fluorescent dye such as HEX, NED, and FAM.

Characterization of each locus was performed using the DNA from 23 individuals that were collected from four locations in Korea: Booyeo, 36°17′37″N, 126°56′57″E; Jain, 35°53′23″N, 128°47′04″E; Sorae: 37°23′00″E, 126°43′00″E; Uljin: 37°02′16″N, 129°23′23″E. Genomic DNAs were extracted according to the standard protocol of Sambrook & Russel (2001). Microsatellites were amplified under the following conditions. The reaction mixtures (total 25 μL) were composed of 0.5 μL of template DNA, 0.7 μL of each primer (10 μM), 1.0 μL of dNTP solution (10 mM), 2.0 μL of $MgCl_2$ (25 mM), 2.5 μL of *Taq* buffer (10×), 0.2 μL of *Taq* DNA polymerase (Promega), and 17.4 μL of DW. PCRs were carried out on GeneAmp 9700 and included the following steps: an initial denaturation at 94 °C for 5 min, 30 cycles of denaturation at 94 °C for 30 s, annealing at a set temperature depending on each locus (Table 1) for 30 s, elongation at 72 °C for 30 s, and final extension at 72 °C for 30 min. PCR products were determined using the Genetic Analyser 3730 (Applied Biosystems), and analysed by GENESCAN 3.7 and GENOTYPER 3.7 (Applied Biosystems).

We designed primers for 17 microsatellites from the sequences of 48 positive clones. Based on the results of characterization, 11 loci were identified as polymorphic (Table 1). Tests of pairwise linkage disequilibrium by contingency exact test using GENEPOP 3.4 (Raymond & Rousset 1995) showed that there was no significant linkage disequilibrium except between ANS107 and ANS121 ($P < 0.0001$). The observed heterozygosity and expected heterozygosity were calculated using ARLEQUIN 3.01 (Excoffier *et al*. 2005), and the Hardy–Weinberg equilibrium (HWE) was tested by GENEPOP 3.4 (Raymond & Rousset 1995). Significant departures from HWE were observed in the case of ANS014, ANS107, ANS113, and ANS121 (Table 1).

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References

Chae J-Y (1999) Re-emerging *Plasmodium vivax* malaria in the Republic of Korea. *Korean Journal of Parasitology*, **37**, 129–143.

Excoffier L, Laval G, Schneider S (2005) ARLEQUIN, version 3.0: an integrated software package for population genetics data analysis. *Evolutionary Bioinformatics Online*, **1**, 47–50.

Hammond RL, Saccheri IJ, Ciofi C *et al.* (1998) Isolation of microsatellite markers in animals. In: *Molecular Tools for Screening Biodiversity* (eds Karp A, Isaac PG, Ingram DS), pp. 279–285. Chapman & Hall, Weinheim, Germany.

Raymond M, Rousset F (1995) GENEPOP (version 1.2): population genetics software for exact tests and ecumenicism. *Journal of Heredity*, **86**, 248–249.

Ree H-I (2005) Studies on *Anopheles sinensis*, the vector species of vivax malaria in Korea. *Korean Journal of Parasitology*, **43**, 75–92.

Rozen S, Skaletsky HJ (2000) PRIMER 3 on the WWW for general users and for biologist programmers. In: *Bioinformatics Methods and Protocols: Methods in Molecular Biology* (eds Krawetz S, Misener S), pp. 365–386. Humana Press, Totowa, New Jersey, USA.

Sambrook J, Russell DW (2001) *Molecular Cloning: a Laboratory Manual*, 3rd edn. Cold Spring Harbor Laboratory Press, New York.