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Full Length Research Paper

Development of microsatellite markers for *Helopeltis theivora* waterhouse (Hemiptera: Miridae)

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Helopeltis theivora **is an important pest of cocoa, tea, mangoes and ornamental plants in Asia. In the effort to control this insect pest effectively, information on its population genetics is needed. Single locus DNA microsatellites are very efficient in being able to provide such information when they are used in the study of the population structures of organisms as they are co-dominant markers. As no such marker is available for this species yet, here we reported in our work, the isolation and development of six of such markers which were found to be polymorphic when tested on samples from three populations of this insect in Peninsular Malaysia.**

Key words: Microsatellite, 5' anchored PCR, heterozygosity, *Helopeltis theivora*.

INTRODUCTION

Helopeltis theivora is known as a pest of cocoa, tea, cashew, mangoes and ornamental plants. The insect feeds on young shoots and fruits and cause dieback of the shoots and stunting of plant growth. Little is known about the genetic background of this mired and not much molecular work has been done. Information on genetic diversity is valued for the management of germplasm, for formulating conservation strategies as well as for planning pest eradication programmes. Molecular markers are the best tools for determining genetic relationships. Numerous molecular markers are now available in entomology (Loxdale and Lushai, 1998) and because microsatellites are highly polymorphic, neutral and show Mendelian inheritance, they therefore represent one of the most powerful tools for population genetic studies (Bruford and Wayne, 1993). Microsatellites have also been analysed extensively in *Drosophila melanogaster* (Bachtrog et al., 1999), *Drosophila simulans* (Hutter et al., 1989) for genetic mapping and for the discrimination of closely related species (Lanzaro et al., 1998).

Single locus microsatellite is a highly informative genetic marker system for analyzing intraspecific phenomena related to both natural genetic substucturing and anthropogenic changes (Presa et al., 2002). Since single locus microsatellite markers are highly variable and co-dominantly inherited, they can be used in genetic diversity, ecological and evolutionary studies. Hybridization method used for the isolation of single locus microsatellite markers, which is still very much in use today, is compared to looking for a needle in a haystack. The method requires the construction of a genomic library using restriction enzymes to generate fragments of the genomic DNA. Fragments within the range of 200 to 600 bp are selected after gel electrophoresis and cloned into competent cells. The clones are then screened for the possible presence of microsatellites through hybridization and subsequently positive clones are sequenced to reveal the microsatellite motifs. The downside of the conventional method is that one would need to screen thousands of colonies to uncover the microsatellites and it could turn out to be extremely tedious and inefficient for species with low microsatellite frequencies (Zane et al., 2002). Recently, more researchers are using enrichment techniques to ensure a higher success rate. Kijas et al. (1994) had used a streptavin-biotin enrichment technique with very impressive results. Others have used modifications of the RAPD technique with equally good results (Cifarelli et al.,

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Table 1. List of 5' anchored PCR primers used for obtaining PCR products for cloning.

No.	Primer	Sequence (5'-3')	Annealing temperature (ºC)
1.	LR1	KKV RVR VCT $(CT)_9$	48
2.	LR2	KKV RVR VRV RVR V$(CT)_6$	50
3.	LR3	GCT AGT GCT $(CA)_7$Y	48
4.	LR7	KKV RVR V$(GA)_{10}$	50
5.	VJ1	NNN NNN NKK VRV RV $(CT)_{10}$	55
6.	VJ2	NNN KKV RVR V $(CTC)_5$	53
7.	VJ3	NNN MMH YHY H $(GGTT)_3$	53

IUB code: K = G/T; N = A/C/G/T; Y = T/C; B = C/G/T; M = A/C; S = C/G.

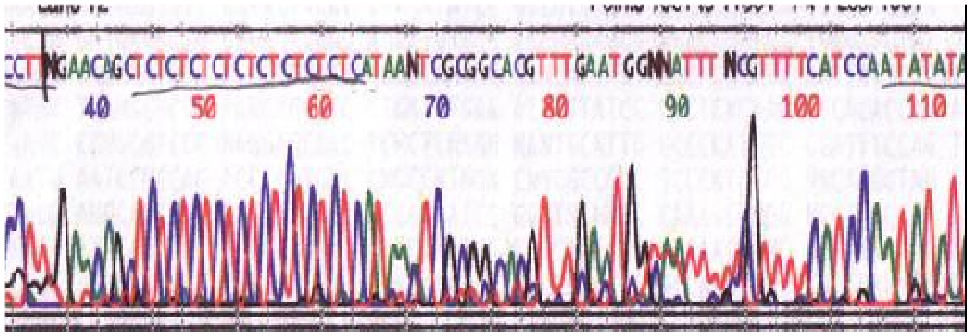

Plate 1. Chromatogram obtained from an automated DNA sequencer showing CT microsatellites from clone HTLR1-20.

1995; Ender et al., 1995). Another enrichment method gave favorable results is 5' anchored PCR. This technique reported by Fisher and Gardner, (1996) is simple, rapid and has been successfully used and reported in previous studies. The isolation of microsatellite markers has been reported in many insect, for example, the parasitic wasp *Cotesia glomerata* (Zhou et al., 2005) and the pollinating wasp *Ceratosolen solmsi* (Yu et al., 2008). Since single locus DNA microsatellite markers have not been previously reported for *H. theivora*, therefore this study is an attempt to be the first to isolate microsatellite markers for this insect.

MATERIALS AND METHODS

DNA extracts from adult *H. theivora* were obtained by using the protocol of Latif et al. (2008) with minor modifications. The primers used with this technique were LR1, LR2, LR3, LR7, VJ1, VJ2 and VJ3 (Table 1) (Kumar et al., 2002). The PCR amplification was carried out in a T3 thermocycler (Biometra) with 10 µl total reaction mixture volume consisting of 20 ng of genomic DNA, 1X PCR buffer (Promega), 250 µm of each dNTPs (Promega), 0.5 µM of primer, 0.5 units of *Taq* DNA polymerase (Promega), 1.0 mM of $MgCl_2$ and deionized distilled water top up to 10 µl. The amplification was performed as follows: 3 min at 96°C for predenaturation, followed by 40 cycles of 10 s each at 96°C for denaturation, optimal annealing temperature of each primer for 10 s and a 30 sec primer extension at 72°C and final extension was carried out at 72°C for 5 min. This was followed by cloning of the PCR product. The cloning of the PCR product was done with a TOPO TA Cloning Kit (Invitrogen, USA).

DNA sequencing was done by using an automated DNA sequencer. Automated sequencing was performed using the Big Dye Terminator Cycle Sequencing Ready reaction Kit (Applied Biosystems, USA) on an ABI PRISM 377 DNA sequencer. The sequencing service was provided by the Institute of Bioscience, Universiti Putra Malaysia. The forward and reverse primers were designed using the software Primer 3, available at http://www.genome.wi.mit.edu/genome software/other/primer3.html. DNA sequences were first edited to remove vector sequences before submission to GenBank. The submission was done through the internet using the online tool Bank IT provided by the GenBank website, http://www.ncbi.nlm.nih.gov/. The designed primer pairs were tested for their ability to amplify polymorphic single locus microsatellites in *H. theivora*. .

The PCR amplification for each of the primer pairs designed was performed in a T3 thermocycler (Biometra) with 10 µl total reaction mixture volume consisting of 10 - 20 ng of genomic DNA, 1X PCR buffer (Promega), 250 µM of each dNTPs (Promega), 0.5 µM of primer, 1.0 unit of *Taq* DNA polymerase (Promega), 1.0 mM of $MgCl_2$ and deionized distilled water to top up to 10 µl. The amplification was performed as follows: 3 min at 96°C for predenaturation, followed by 40 cycles of 10 s each at 96°C for denaturation, optimal annealing temperature of each primer for 10 s and a 30 s primer extension at 72°C and final extension was carried out at 72°C for 5 min.

To assess for polymorphism, 10 individuals of *H. theivora* collected from each of the tea plantations at Bukit Cheeding (Banting), Sungai Palas (Cameron Highlands) and Tanah Rata (Cameron Highlands) Malaysia were genotyped for each locus. Successful primer pairs reproducibly amplified specific products of the expected sizes as judged by electrophoresis on 4% Metaphor agarose gel (FMC, USA) with 20 bp molecular marker (BMA, USA), stained with ethidium bromide (0.1 mg/ml) and visualized using UV light (Plate 1).

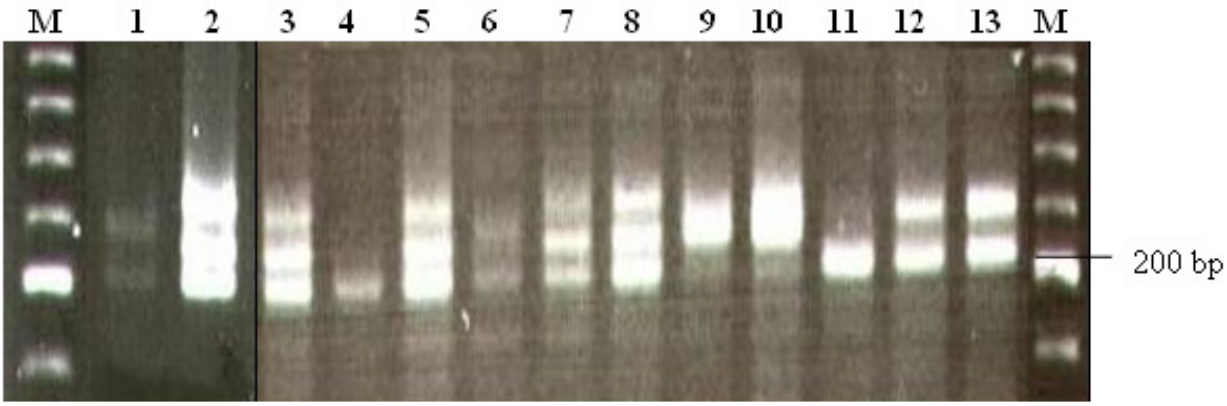

Plate 2. Microsatellite profile of *H. theivora* using primer pair LR7-CT9. Lanes 1 - 4: Microsatellite profiles from Bukit Cheeding (Banting) samples; lanes 5 - 8: Microsatellites profiles from Sungai Palas (Cameron Highlands) samples; lanes 9 - 13: Microsatellites profiles from Tanah Rata (Cameron Highlands) samples; M: 20 bp molecular marker (BMA, USA). PCR products were run on a 4% metaphor agarose gel in 1X TBE buffer at 70 V for 3 h.

Table 2. Number of clones and repeat motif detected by RAMs primers and GenBank accession numbers.

Clone name	Repeat Motif detected	GenBank accession numbers
HTLR1-8	$(CT)_5$	DQ677233
HTLR1-18	$(GA)_7$	DQ677234
HTLR7-6	$(GT)_3,(CT)_{10}$	DQ677235
HTLR1-20	$(CT)_{10}$	DQ677236
HTLR1-21	$(GA)_7$	DQ677237
HTLR1-10	$(CT)_{10}$	DQ677238
HTLR7-8	$(CT)_5$	DQ677239
HTLR7-12	$(GCA)_2,(CT)_2$	DQ677240
HTLR7-15	$(GA)_7$	DQ677241
HTLR7-17	$(GA)_4$	DQ677242
HTVJ2-13	$(CCT)_4$	DQ677243
HTVJ2-14	$(CCA)_5$	DQ677244
HTLR7-18	$(GA)_{11}$	DQ677245
HTLR7-24	$(CT)_{14}$	DQ677246
HTLR7-14	$(GA)_7$	DQ677247
HTLR7-9	$(CT)_9$	DQ677248
HTVJ2-10	$(CCT)_4$	DQ677249
HTVJ2-12	$(GGA)_4$	DQ677250

RESULTS AND DISCUSSION

In this study, almost 400 clones were isolated (Plate 2) and a total of twenty clones were sent for automated DNA sequencing. A total of 18 microsatellite regions were detected. Table 2 shows the repeat sequences that were obtained and the GenBank accession numbers for each of them. Of the 18 primer pairs designed and tested, twelve were considered to be uninformative either because of amplifications at more than one region, lack of polymorphism or difficulties in scoring the bands. Nevertheless, all the 18 microsatellite-containing sequences obtained from this study were deposited in GenBank and given accession numbers as listed in Table 2 as they may all prove to be useful if other PCR conditions were tested for the optimizing the bands produced. For each of the six informative loci, the allelic frequencies and the expected and observed heterozygosities of the pooled data were calculated with the assistance of the POPGENE ver.1.31 software (Yeh and Boyle, 1997). All six of the useable microsatellite loci were found to be polymorphic based on the analysis of 30 samples of *H. theivora* and their characteristics are presented in Table 3. For all six loci, the observed heterozygosities were

Table 3. Primer sequences (5'- 3') and the characteristics of six *H. theivora* microsatellite loci. The number of alleles and the observed and expected heterozygosities were based on 30 individual insects.

Locus	Repeat motif	Primer sequence	T_a (°C)	Allele size (bp)	N_a	H_0	H_E	GenBank Accession no.
LR1-CT2	$(GA)_7$	F: GAGACGATTGGTGTTACTAC R: TTGAAACCTCTCTCTCTCT	58	245	9	0.30	0.45	DQ677234
LR7-CT4	$(GT)_3(CT)_{10}$	F: GTCATTAATAACGCTCCAG R:GATCAAAACTTCAAGTCAGC	58	236	4	0.23	0.40	DQ677235
LR7-CT9	$(GA)_7$	F: CGAGAGAGAGAGAGATGATA R:TTAAGTACTAGCTGGATTCG	58	248	6	0.00	0.31	DQ677241
VJ2-CT12	$(GGA)_4$	F: CACAGAGAGAAGCCACAT R:ATTCGCCCTTTGGTTCAA	45	239	7	0.40	0.74	DQ677250
LR7-CT24	$(CT)_{14}$	F: AATAGGTAAGGAGCAGAGTT R:TGTCAGGGGAGAGAGAGAGAG	42	213	9	0.15	0.77	DQ677246
LR7-CT14	$(GA)_7$	F: TGCGCGGGAGAGAGAG R:AAGGAGCAGAGTTTCTCTA	45	200	7	0.10	0.76	DQ677247

T_a, annealing temperature; N_a, number of allele; H_O, observed heterozygosity; H_E, expected heterozygosity.

considerably lower than the expected values. This could be due to the small number of individuals; 10 individuals per population and the pooling of data from three populations. However, this study was done to design suitable microsatellite primer pairs for this mirid insect and to assess the presence of polymorphisms for them. They could now be used in large scale population genetic studies of *H. theivora* in the future.

Conclusion

This study showed that 5'anchored PCR technique was suitable and efficient for isolating single locus DNA microsatellites for *H. theivora*. A total of 6 polymorphic microsatellite markers were isolated successfully. This study is important in the quest to provide a sufficient number of codominant genetic markers for studying the population genetic structure of *H. theivora* in Asia.

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