ONLINE RESOURCES

Development of single locus DNA microsatellite markers in *Oryctes rhinoceros* (Linnaeus) using 5′ anchored RAMs-PCR method

G. MANJERI[1], R. MUHAMAD[1]*, Q. Z. FARIDAH[2] and S. G. TAN[3]

[1]*Department of Plant Protection, Faculty of Agriculture,* [2]*Department of Biology, Faculty of Science and* [3]*Department of Cell and Molecular Biology, Faculty of Biotechnology and Biomolecular Sciences, Universiti Putra Malaysia, 43400 UPM, Serdang, Selangor, Malaysia*

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Introduction

Oryctes rhinoceros, commonly known as rhinoceros beetle, is an important pest in oil palm plantations. The presence of this pest in replanting sites as early as six months after replanting has alarmed planters due to the possibility of increased crop damage (Samsuddin *et al.* 1993; Kamarudin and Wahid 1997). Being a nocturnal animal with a destructive feeding habit, it is difficult to eliminate this pest (Young 1986). Pheromone trapping using a species-specific-aggregation pheromone is commonly used to trap *O. rhinoceros* in replanting sites (Hallet *et al.* 1995). However, not all population of *O. rhinoceros* in the field were observed to be attracted by it. This suggests the possibility of a cryptic species complex occurrence in this insect. To investigate this notion, a thorough study utilizing molecular markers is necessary. An example of a powerful marker with proven capability for resolving such issues is the single-locus DNA microsatellite marker. Being codominant, multiallelic and highly polymorphic, microsatellites are a powerful and promising genetic marker, suitable for precise discrimination of closely related individuals (Smouse and Chevillon 1998). A previous study on the genetic variation of *O. rhinoceros* had highlighted the necessity of further studying this pest species using single-locus microsatellite DNA markers as *O. rhinoceros* were reported to exhibit possible occurrence in two groups (Manjeri *et al.* 2011). Therefore, with interest to further study the population genetic structure of *O. rhinoceros*; this study was carried out to isolate sufficiently novel single-locus microsatellite markers for the insect. The isolation was carried out based on the 5′-anchored polymerase chain reaction (PCR) technique (Fisher *et al.* 1996) using anchored randomly amplified microsatellite (RAM) primers (Kumar *et al.* 2002; Hoh *et al.* 2008).

Materials and method

Genomic DNA was extracted from *O. rhinoceros* beetle using the Wizard Genomic DNA Purification kit (Promega, Madison, USA) according to the manufacturer's protocol for animal tissue genomic DNA isolation with minor modifications. Tissues from the thorax and head of the beetle were used to avoid contamination. Extracted DNA was quantified using a NanoDrop ND-1000 Spectrophotometer (Thermo Fisher Scientific, Wilmington, USA). Fifteen RAMs primer were tested using the extracted DNA. However, only nine primers with clear banding profile were chosen. PCR was performed in a total volume of 10 μL per reaction containing 25 ng of genomic DNA, 2.5 mM $MgCl_2$, 1× PCR Buffer (10 mM Tris-HCl, 50 mM KCl and 0.1 Triton R_X-100), 0.4 mM dNTPs mix, optimized specific concentration of primer, 3 U *Taq* DNA polymerase (Promega, Madison, USA) and topped with deionized distilled water up to 10 μL. Amplifications were performed in a Techne TC-412 thermal cycler (Burlington, USA) with an initial 3 min of predenaturation at 96°C, followed by 40 cycles of 20 s denaturation at 95°C, 20 s annealing at optimized temperature for each primer and 35 s extension at 68°C. A final extension step of 68°C for 5 min was included. PCR product, 5 μL was electrophoresed on horizontal 2% agarose gel (w/v) at 70 V in 1× TBE buffer (0.045 M Tris-borate and 1 mM EDTA, pH 8). A 100-bp extended ladder (Fermentas, Burlington, USA) was used as a reference. Gels were stained in 0.1 μg/μL ethidium bromide and visualized over UV light and captured using Alpha®Imager 2200 (Alpha Innotech, San Leandro, USA) system. PCR

*For correspondence. E-mail: ritamuhamad@yahoo.com.

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products were then subjected to nucleic acid extraction using the GF-1 PCR Cleanup kit (Vivantis Technologies, Sham Alam, Malaysia).

Purified products were cloned using the TOPO TA Cloning kit (Invitogen, Carlsbad, USA). Procedures were according to the manufacturer's protocols with slight modification observed for duration. Fifteen putative recombinant clones from each primer were randomly picked and subcultured overnight in LB broth. Cells were then harvested and subjected to plasmid extraction using PureLink Quick Plasmid Miniprep Kit (Invitrogen) according to the manufacturer's protocol. Plasmids were sent to First Base Laboratories (Seri Kembangan, Malaysia) for forward and reverse automated DNA sequencing using Big Dye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems, Foster City, USA) on the ABI 3730XL DNA sequencer. Forward and reverse sequences were aligned and vector regions were removed. Repeat motifs were identified using the simple sequence repeat identification tool (SSRIT) (Temnykh *et al.* 2001). Microsatellite regions were deposited in GenBank using BankIT tool (https://www.ncbi.nlm.nih.gov/). Primer pairs were designed to amplify the microsatellite region using the program Primer 3 (Rozen and Skaletsky 2000).

The designed primer pairs were optimized and tested for polymorphism. Successfully optimized primer pairs were characterized using 60 *O. rhinoceros* individuals collected randomly from several oil palm plantations. PCR was performed in 10 μL reaction volume which consisted of 15 ng of genomic DNA, 3.5 mM $MgCl_2$, 1× PCR Buffer, 0.4 mM of dNTPs Mix, 0.3 μM of each primer, 1 U of *Taq* DNA polymerase (Promega) and deionized water using Techne TC-412 thermal cycler. PCR cycle had an initial 3 min of predenaturation at 96°C, followed by 35 cycles of 20 s denaturation at 95°C, 20 s annealing at optimized temperature for each primer and 35 s extension at 68°C. A final extension step of 68°C for 5 min was included. PCR products were subjected to electrophoresis on 8% (w/v) vertical polyacrylamide gel using 1× TBE buffer (0.045 M Tris-borate and 1 mM EDTA, pH 8). A 20-bp extended ladder (Cambrex, Rockland, USA) was used for sizing. Gels stained in 0.1 μg/μL of ethidium bromide were viewed and captured using Alpha®Imager 2200 (Alpha Innotech, San Leandro, USA) system. Alleles were scored using AlphaEaseFC™ v4.0 software (Alpha Innotech). Null allele frequencies were estimated using Microchecker v2.2.3 (Van Oosterhout *et al.* 2004). Genetic diversity data were obtained using Popgene v1.32 (Yeh *et al.* 1997) and Genepop v4.0.10 (Rousset 2008).

Results and discussion

Nine 5′anchored RAMs primers with clear banding profiles were used for the isolation of microsatellite repeat motifs in *O. rhinoceros*. Table 1 shows the selected 5′anchored RAMs primers with their respective annealing temperature and primer concentration. Seventy-six clones were chosen for automated sequencing and from these, 50 clones revealed sequences with unique repeat motifs. Meanwhile, eight clones were redundant copies and 18 clones could not be sequenced due to template purity problems.

Hundred and eighty microsatellite repeat motifs were identified and the isolated repeat motifs consisted of 151 perfect repeats, 14 interrupted repeats, 10 compound repeats, three interrupted compound repeats, one interrupted complex repeat and one complex repeat. Repeat motifs were present in both the internal region and the 5′ and 3′ terminal of the sequences. Isolated motifs were made up of different core units ranging from mononucleotide to hexanucleotide repeats. However, dinucleotide (31.67%) and pentanucleotide (32.22%) units were isolated most.

Hundred and forty-four microsatellite primer pairs were designed complimentary to the conserved flanking regions. Some primers were designed to amplify more than one repeat motif region due to the proximity between repeat motifs. Subsequent to thorough optimization of all primers, 83 primer pairs amplified microsatellite regions. Of this, 32 primer pairs were polymorphic and 51 were monomorphic. Meanwhile, the remaining 61 primer pairs failed to amplify any microsatellite regions being deemed uninformative.

All the 32 polymorphic markers that were characterized by genotyping 60 *O. rhinoceros* beetles produced single-locus amplifications throughout. Table 2 presents the char-

Table 1. Selected 5′anchored RAMs primers with their respective annealing temperature (T_a) and optimized primer concentration.

Primer	Primer sequence (5′–3′)	T_a (°C)	Primer concentration (μm)
LR4	GCA CAT GCA R$(TG)_7$	55.0	0.3
LR5	GAT GCG ATR$(CA)_7$	55.0	0.5
LR7	KKV RVR V$(GA)_{10}$	53.0	0.4
BP8	KKY HYH YHY $(GTT)_5$	50.0	0.5
BP10	KKD RDR D$(TC)_{10}$	43.6	0.4
BP11	KKY HYH Y$(CAG)_5$	50.0	0.4
BP14	KKB YSS $(GATA)_5$	39.0	0.4
SC3	NNN NNN NNN MMH RVH RV$(GTC)_4$	39.0	0.4
VJ2	NNN KKV RVR V$(CTC)_5$	38.4	0.4

Table 2. Characteristics and descriptive statistics of 32 polymorphic single-locus microsatellite markers of *O. rhinoceros* which were screened through 60 individuals.

Locus	Primer sequence (5′–3′)	Repeat motif	T_a (°C)	N_A (size, bp)	H_O	H_E	*P* value	Accession number
OrBP8M-3-1	F: GGCCTTTCTCGTTGTTGT R: TATAGCCAAGTCGCTGTTCC	$(GTT)_5$ $(AGAAAA)_2$ $(CTGCA)_2$ $(GA)_4$	45.5	2 (256–280)	0.5370	0.4907	0.2422	HM068025
OrBP8M-3-2	F: AGGACTGTGGCGTAATATGG R: TAGTATGGCCGGTAATCTGG	$(AATC)_3$	47.3	3 (308–328)	0.6964	0.6588	0.0005*	HM068025
OrBPM8-3-4	F: TTGGATCAATGCTCTGCTTG R: TGGCACCTGGTTGTTGTTG	$(CAA)_6$	49.0	8 (151–187)	0.8793	0.7612	0.0000*	HM068025
OrBP11M-1-1	F: ACAATGGAATGACACATACG R: GACGCGAATACCAACAAATC	$(TTAAT)_2$	47.0	4 (163–183)	0.4333	0.3716	0.4889	HM068033
OrBP11M-3-1	F: TACAAAGTTTACGGCCAATC R: CGTCCCAATGCCTATTTC	$(AAGAA)_2$	45.3	2 (152–162)	0.1500	0.1399	1.0000	HM068035
OrBP11M-3-2	F: AAACCATCCGAAAGAAGATG R: TTGTAGATAGGAGACGAGGAAG	$(ACAT)_3$	47.0	2 (170–190)	0.2167	0.2206	0.6992	HM068035
OrBP11M-7-2	F: CCGGCCATACTATAAAGACG R: TTTGTTTCGGGTAAAGCACAG	$(TA)_3C(TA)_{10}$	47.0	3 (214–248)	0.0339	0.1872	0.0013*	HM068037
OrBP11M-8-2	F: CGCAAACCGCACTTTACCAC R: CAACGCTCGTGCTCCTACC	$(TTCAG)_2$	55.0	2 (150–165)	0.0500	0.0492	0.0000*	HM068038
OrBP11M-9-1	F: ATCAGCGCCACCAAACTTAC R: AACCGTTTTCGGCCATATC	$(TTTGGA)_2$	50.6	2 (258–264)	0.0167	0.4637	0.0000*	HM068039
OrBP14M-9-2	F: GCTATCCAGACGGAAAACCT R: CACTACTGATCGTCCTAGTTCTGT	$(TATTAC)_2$	52.4	4 (115–133)	0.5254	0.4336	0.9974	HM068046
OrSC3M-4-4	F: TCGTTTCGGCAGTAATATGG R: TGTACGATGAGCGATACTGG	$(TA)_6$	51.5	2 (223–233)	0.2542	0.4573	0.0189	HM068027
OrSC3M-5-1	F: CAAGGCCATCGAACTCTCC R: CAAGAAACACCGACATTATCAAC	$(GAAAT)_2$	51.0	4 (254–269)	0.3500	0.6784	0.0034	HM068028
OrSC3M-6-3	F: TCGTTGCATGTATGGTTTCG R: GTTGATAATGTCGGTGTTTCTTG	$(ATGT)_4$	48.5	3 (224–240)	0.0862	0.0841	0.0000*	HM068029
OrSC3M-9-2	F: TCGAAGCGAGGAGAATATCG R: CCAGACAGCACAACGTCAAC	$(AG)_4AC(AG)_2$	50.8	2 (281–285)	0.7455	0.4719	0.0592	HM068030
OrVJ2M-1-2	F: CTTTGGTCGTCGCTATTTGC R: GGCTTCACTTGGAACTCCTG	$(TATTA)_2$	49.5	2 (264–274)	0.1373	0.1291	1.0000	HM068018
OrVJ2M-1-3	F: GGTGTTCATGCCTTCCTC R: AAAGATGACCGCCTATTATTG	$(TA)_4$	49.5	3 (197–205)	0.7407	0.5291	0.0651	HM068018
OrVJ2M-4-2	F: AGCACATATCATCGGGTCAAG R: CGGAAGACTCAAGAAGAAACG	$(AATAAA)_2 \ldots (ATA)_5$	50.8	2 (184–190)	0.2807	0.5005	0.7584	HM068019
OrVJ2M-5-3	F: ATTATCCCGTCTGCAAGAAC R: TTAAAGTCCGGGAGCAAGC	$(TAAG)_2$ $(GAAC)_2$ $(TTAA)_2$	49.5	2 (234–242)	0.0870	0.0841	0.0000*	HM068020

Table 2 (*contd.*)

Locus	Primer sequence (5′–3′)	Repeat motif	T_a (°C)	N_A (size, bp)	H_O	H_E	P value	Accession number
OrVJ2M-7-4	F: CTTTTGACCATTGCCTTTGG R: CAGCCCATTTGAATTTTGTG	$(TAAA)_2(ATTT)_2$	48.2	2 (218–226)	0.1525	0.1705	1.0000	HM068021
OrLR4M-1-2	F: AAAGCAACCGAGTTCGTTC R: GCAATAAACAGATGGCAATG	$(TA)_3N_8(TA)_3N_2(TA)_3$	48.4	6 (242–276)	0.5439	0.7639	1.0000	HM068058
OrLR4M-1-3	F: AAAGTATTTAGCGGATTGCC R: TGGGATTGCCGACTCTTC	$(CGAAA)_2$	46.2	2 (266–276)	0.0000	0.0958	0.0000*	HM068058
OrLR4M-2-5	F: TCGAGATAATCAACGTGAGAG R: GGACGCCATTATGTTAAACG	$(AG)_4$	50.2	2 (176–186)	0.1017	0.0973	1.0000	HM068059
OrLR4M-2-6	F: CTAGTACGCGGTGAGACCTAG R: TGCACATGCAGTGTGTGTG	$(AC)_8$	52.8	2 (113–119)	0.9333	0.5020	0.0000*	HM068059
OrLR4M-3-2	F: GCCGAATTGTGAGATGGTTC R: CTTTCAATGTTGCCATCAGC	$(AAACG)_2$	47.5	2 (198–208)	0.7273	0.4911	0.0104	HM068060
OrLR4M-3-3	F: AACCGGAATAAACTCGTCGTC R: CGCATAGTGAGCACAGGAAC	$(AC)_8$ $(GT)_5$ $(TGTAG)_2$	50.2	2 (190–195)	0.5849	0.4695	0.1345	HM068060
OrLR4M-4-5	F: GTAACTAAGCCGGAGGTTCG R: TGCACATGGGAGTGTGTG	$(AC)_8$	52.8	2 (167–171)	0.7966	0.5037	0.0043	HM068061
OrLR4M-6-1	F: GGATGTGCAATGTGTGTG R: CGTTGTCGAACAGTAAACG	$(TG)_7$ $(ATTAA)_2$	48.8	2 (168–170)	0.7119	0.4624	0.0018	HM068063
OrLR4M-7-2	F: CGAACCTCCGGCTTAGTTAC R: AAACCCACAGATCACATTGC	$(AAAAT)_2$	47.0	2 (190–195)	0.3061	0.5043	0.0101	HM068064
OrLR4M-8-2	F: AGGCAGCGGCAGTAAGTG R: TTAAGCCCTCGTTGGACAAG	$(ACGGA)_2$	46.0	2 (199–209)	0.4000	0.3229	0.4448	HM068065
OrLR4M-8-3	F: TGTTACCGTGTTTGATTCATCC R: CAATGTCGAGCCTAGCCAAC	$(T)_{10}$	48.5	3 (217–225)	0.2245	0.3516	0.3828	HM068065
OrLR4M-8-4	F: GTTGGCTAGGCTCGACATTG R: CTAACGACGGGTAGTGATTGG	$(ATTGG)_2$	51.5	2 (244–249)	0.0370	0.1059	0.0000*	HM068065
OrLR4M-8-5	F: TTCGCTGACCGACACTTAAC R: ACTGCCATGCCTATCAACAC	$(A)_4T(A)_{10}$	50.5	6 (168–174)	0.6780	0.6764	0.0000*	HM068065

T_a, annealing temperature; N_A, number of alleles; H_O, observed heterozygosity; H_E, expected heterozygosity; P, possibilities to fit to HWE using an exact test. *Deviation from HWE after Bonferroni correction ($P < 0.05$, minimum adjusted alpha = 0.00156).

acteristics and descriptive statistics of all the 32 polymorphic single-locus microsatellite markers isolated for *O. rhinoceros*. No null alleles, stutter bands or large allele dropout were detected for the entire 32 locus. A total of 89 alleles were successfully identified and the number of alleles per locus ranged from two to eight with an average of 2.78 alleles per polymorphic locus. Allele size ranged between 113 bp and 328 bp. The observed heterozygosity values ranged from 0.000 to 0.9333 and the expected heterozygosity value ranged from 0.0492 to 0.7639. Observed heterozygosities were higher than expected heterozygosities in 20 of the isolated microsatellite markers. After the sequential Bonferroni correction (Rice 1989) (minimum adjusted alpha = 0.00156), 11 loci revealed significant deviation from Hardy–Weinberg equilibrium. Moreover, no significant linkage disequilibrium was detected for any pairs of loci indicating that the markers were independent.

Conclusion

The development of these 32 polymorphic single-locus DNA microsatellite markers for *O. rhinoceros* has brought us one step closer to further understand the population genetic structure of this important agricultural pest of the tropics. Utilizing the 5′ anchored RAMs-PCR technique, a large number of polymorphic single-locus DNA microsatellite markers had been isolated within a limited time frame. These markers have now laid path for further population genetic studies on *O. rhinoceros* in oil palm plantations worldwide.

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