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Isolation by the 5′ anchored PCR technique and characterization of eighteen microsatellite loci in horseshoe crab (*Tachypleus gigas*)

LIEW PUI LING, ABU BAKAR ADIBAH, SOON GUAN TAN*, ANNIE CHRISTIANUS and QAMARUZ ZAMAN FARIDAH

Universiti Putra Malaysia, UPM Serdang, Selangor, 43400, Malaysia

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The horseshoe crab, a well-known 'living fossil', is represented by two species (*Tachypleus gigas* and *Carcinoscorpius rotundicauda*) in peninsular Malaysia. One of these, *T. gigas* inhabits shallow marine waters and migrates to intertidal sandy beaches for breeding during high tides at every full and new moon throughout the year (Hajeb *et al.* 2009). This species is being used as bait by local fishermen to catch crabs or shrimps by netting or cage fishing methods. Due to its low commercial value in comparison to other marine economic species such as fishes, crabs and shrimps, most of the animals caught in fishing nets or cages are released immediately back to sea by fishermen or left to die in the sun. In certain states of Malaysia, such as Kelantan, Penang and Johore, adult female horseshoe crabs are sold to local seafood restaurants and in fish markets due to consumption of their eggs by the local people.

Horseshoe crab populations are rapidly declining around the world due to various reasons such as water pollution, loss of their living and spawning habitats and human exploitation of adult horseshoe crabs (Li *et al.* 2009; Shin *et al.* 2009). Assessing the genetic variability of horseshoe crab is important as it has direct benefit for the conservation and management of wild populations of the species. However, there is no information available on the population genetic structure and diversity of *T. gigas* in Malaysia. To address this research need, we have isolated and developed the first set of polymorphic microsatellite markers for *T. gigas* by using the 5′ anchored PCR technique.

A total of 130 individuals of horseshoe crabs were collected from five locations along the coastal areas of peninsular Malaysia, namely Port Dickson (Negeri Sembilan), Pantai Bersih (Penang), Sungai Muar (Johor), Pantai Balok (Pahang) and Kg. Sungai Pulai (Selangor). Genomic DNA was extracted from muscle tissue of *T. gigas* by using the protocol described by Winnepennincks *et al.* (1993) with a minor modification to the CTAB buffer (2% w/v CTAB, 1.4 M NaCl, 20 mM EDTA, 100 mM Tris-HCl, pH 8.0). The concentration and purity of the DNA were quantified using a NanoDrop ND-1000 Spectrophotometer (Thermo Fisher Scientific, Wilmington, USA).

A modification of the 5′ anchored PCR technique of Fisher *et al.* (1996) was used for the isolation of microsatellite loci from *T. gigas*. The modification was made by choosing 5′ anchored primers with a relatively long length in order to capture longer microsatellite flanking sequences, thus allowing more flexibility for primer design. Six selected 5′ anchored ISSR primers (Kumar *et al.* 2002; Hoh 2005) were used for the PCR amplification as listed in table 1. The PCR amplification was performed in a total volume of 10 μL containing 1× PCR buffer (10 mM Tris-HCl, 50 mM KCl and 0.1 Triton® X-100), 3.75 mM $MgCl_2$, 0.3 mM dNTPs Mix, 0.15 U *Taq* DNA polymerase (Promega, Madison, USA), 0.5 μM primer (First Base, Seri Kembangan, Malaysia), 100 ng of genomic DNA and deionized water. The amplification was performed in a Biometra® TProfessional thermocycler (Horsham, Pennsylvania, USA) with an initial 3 min of predenaturation at 95°C, followed by 40 cycles of denaturation at 95°C for 30 s, annealing for 30 s, 72°C for 30 s and a final extension step at 72°C for 5 min. The PCR products were separated by electrophoresis on 2% TBE agarose gel and visualized by UV-light. The resultant products were then purified using the GF-1 PCR Clean-Up kit (Vivantis, Sham Alam, Malaysia).

The purified products were subsequently cloned into TOPO TA cloning vector and transformed into One Shot Chemically Competent *Escherichia coli* cells according to the manufacturer's instructions (Invitrogen, Carlsbad,

*For correspondence. E-mail: sgtan_98@yahoo.com; sgtan@biotech.upm.edu.my.

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Table 1. Six selected 5′ anchored ISSR primers.

Primer name	Sequence (5′ to 3′)	T_a (°C)
BP1	NNNMMHVHVHVH$(TG)_{10}$	55
BP2	NNNNNKKVWBDBDBDB$(AC)_{10}$	55
BP11	KKYHYHY$(CAG)_5$	50
BP13	KKBSBSBSB$(CT)_6$	50
LR1	KKVRVRV$(CT)_{10}$	50
RAM1	YHY$(CCA)_5$	54

T_a, annealing temperature; IUB code: N, A/G/T/C; M, A/C; H, A/C/T; V, A/C/G; K, G/T; W, A/T; B, C/G/T; D, A/G/T; Y, T/C; S, C/G; R, A/G.

USA) with minor modification. Ten recombinant clones that appeared as white colonies from each primer were selected randomly for plasmid extraction. Plasmids were extracted using PureLink™ Quick Plasmid Miniprep kit (Invitrogen, Carlsbad, USA) and sent to First Base Laboratory (Seri Kembangan, Malaysia) for sequencing. Fifty-one of the 60 clones sequenced contained two expected microsatellite repeat regions at the 5′ and 3′ terminal ends. In addition, 23 of the 51 clones contained one or more additional internal microsatellites located in between the 5′ and 3′ terminal ends which increased the total number of microsatellite loci isolated. Such internal microsatellite sequences need not have the same repeat units as the anchored primers unlike those at the terminal ends. Primers flanking the microsatellite repeat motifs were designed using the online software, PRIMER 3 (Rozen and Skaletsky 2000) with primer lengths ranging from 18 to 24 nucleotides and PCR product sizes ranging from 150 to 300 bp.

A total of 52 primer pairs were designed and tested, with 18 of these showing polymorphisms, while the rest were monomorphic, failed to amplify or gave inconsistent banding profiles. The PCR amplification was performed in a total volume of 10 μL containing 1X PCR buffer (10 mM Tris-HCl, 50 mM KCl and 0.1 Triton® X-100), 2.0–3.75 mM $MgCl_2$,

Table 2. Characterization of 18 microsatellite loci for *T. gigas*.

Locus	Accession no.	Primer sequence (5′ →3′)	Repeat motif	T_a (°C)	*N* (size, bp)
PLbp1-2-2	GU458322	F: TCAGGGTTGTTTAGTCCTCCAC R: GCTTAACCGCTTCTCAATGG	$(TTAGTG)_2$	60	2 (216–234)
PLbp1-3-2	GU458323	F: CACACCGAGGGGAAAATAAC R: ACTTCCGGCTTGTGTTGTG	$(CAAATA)_2$	60	2 (264–288)
PLbp2-1-2	GU458330	F: GGGCGTTGCAGTTAGATGG R: CCTTGACGGAATCGAGTGG	$(TTTAT)_2(CTAT)_3$	60	5 (200–255)
PLbp2-2-2	GU458331	F: TTGTTCGCCCAAATAAGC R: AAACATCCACCAGAAAGGAC	$(TC)_8TG(T)_7$	57	4 (236–264)
PLbp11-3-1	GU592164	F: TTCTCTCCAGCAGCAGCAG R: GGCATTCGCAAAGAAGCTC	$(CAG)_5$	50	2 (270–279)
Plbp11-9-2	GU592170	F: GGATAACAATCCCCACAGC R: TGTATACCAGCAGCAGCAG	$(CTG)_5$	48	2 (210–216)
PLbp11-10-2	GU592171	F: GTGCAACAATCGCGGTAGG R: GGTCCATCAGCAGCAGCAG	$(CTG)_5$	58	2 (261–294)
PLbp13-1-2	HM021773	F: ACTAAACTCCTGCCAAGG R: CTTTGTTAGATTGTGTAGGC	$(TTCG)_3$	54	2 (160–164)
PLbp13-5-3	HM021776	F: GCTTCGCACTTTGGTGTC R: TTTGGATGGAGGTGTTCG	$(AGTTGA)_2$	54	2 (180–192)
PLbp13-5-5	HM021776	F: GATGGCTCTCAAATAGCTTCG R: CCGTGATTGTTTCAGTTCTGTAG	$(T)_{11}$	58	3 (261–285)
PLbp13-5-7	HM021776	F: GGTATCCCTTCATTCGTCAAG R: TCCAAACCAACCCTCTGTTC	$(TGTTTT)_3$	58	4 (186–258)
PLbp13-5-8	HM021776	F: AGAACAGAGGGTTGGTTTGG R: GGTGTGGGGCTCTCTCTC	$(AG)_7$	58	3 (156–188)
PLbp13-10-1	HM021781	F: GGCGGCTCTCTCTCTCTC R: TGCCTTTATGTTCCTTCTCTG	$(CT)_{15}(T)_{10}$	54	3 (285–300)
PLbp13-10-5	HM021781	F: CCACTGAGGCGTACATAAAGC R: CCATTGTTTGCACTTATCACG	$(AACA)_3$	52	3 (240–252)
PLbp13-10-6	HM021781	F: TCTCCATCTATGTGTTTCTG R: ATTGGCATCTGTTCAGTC	$(ATTAAA)_2$	56	2 (282–294)
PLlr1-9-2	HM032036	F: GTGGAAAATAGACCTTGC R: GGCAAAACTCTCTCTCTC	$(AG)_{10}$	52	6 (250–300)
PLram1-2-1	HM038535	F: CAACGGAAGGTACGACTGAC R: GCAGTAGCGTGGCAGTATTC	$(TTG)_4$	58	2 (213–228)
PLram1-10-1	HM038540	F: ACACTCTTACGGCTGAGTTG R: GTGCTCATTTAGTCGGTGAG	$(TTG)_4$	60	2 (231–249)

T_a, annealing temperature; *N*, number of alleles.

Table 3. Heterozygosity in five populations based on 18 microsatellite loci.

Locus	Port Dickson ($n = 25$)		Pantai Bersih ($n = 25$)		Sungai Muar ($n = 25$)		Pantai Balok ($n = 25$)		Kg. Sungai Pulai ($n = 30$)	
	H_O	H_E	H_O	H_E	H_O	H_E	H_O	H_E	H_O	H_E
PLbp1-2-2	0.520*	0.429*	0.520*	0.429*	0.560*	0.490*	0.680	0.481	0.633*	0.494*
PLbp1-3-2	0.480*	0.372*	0.600*	0.429*	0.760	0.481	0.520*	0.393*	0.667	0.452
PLbp2-1-2	0.160	0.322	0.200	0.406	0.160	0.376	0.160	0.349	0.200	0.435
PLbp2-2-2	0.080	0.451	0.000	0.340	0.040	0.220	0.000	0.346	0.100	0.297
PLbp11-3-1	0.320*	0.327*	0.200*	0.184*	0.280*	0.350*	0.435*	0.487*	0.233*	0.305*
PLbp11-9-2	0.083	0.337	0.160*	0.216*	0.217*	0.198*	0.042	0.191	0.250*	0.275*
PLbp11-10-2	0.640*	0.444*	0.040	0.040	0.960	0.509	0.960	0.509	0.933	0.506
PLbp13-1-2	0.000	0.503	0.000	0.490	0.000	0.372	0.000	0.444	0.000	0.452
PLbp13-5-3	0.400*	0.327*	0.520*	0.393*	0.320*	0.274*	0.333*	0.284*	0.433*	0.345*
PLbp13-5-5	0.640	0.679	0.600	0.589	0.800	0.575	0.720	0.598	0.833	0.549
PLbp13-5-7	0.080	0.545	0.000	0.503	0.000	0.444	0.240	0.562	0.067	0.532
PLbp13-5-8	0.160	0.465	0.360	0.571	0.125	0.451	0.040	0.334	0.448	0.496
PLbp13-10-1	0.040	0.530	0.000	0.510	0.227	0.587	0.000	0.327	0.033	0.332
PLbp13-10-5	0.080	0.389	0.200	0.425	0.000	0.216	0.000	0.000	0.000	0.000
PLbp13-10-6	0.880	0.503	0.542*	0.403*	0.560*	0.411*	0.560*	0.444*	0.533*	0.398*
PLlr1-9-2	0.160	0.376	0.360*	0.371*	0.250*	0.228*	0.217	0.409	0.300*	0.324*
PLram1-2-1	0.000	0.000	0.000	0.082	0.040	0.040	1.000	0.510	0.133*	0.127*
PLram1-10-1	0.480*	0.372*	0.360*	0.301*	0.000	0.000	0.640*	0.444*	0.700	0.463

n, Sample size; H_O, observed heterozygosity; H_E, expected heterozygosity. *Locus conforming to HWE ($P > 0.05$).

0.25 mM dNTPs mix, 0.1 U *Taq* DNA polymerase (Promega, Madison, USA), 0.3–0.5 μM of each forward and reverse primers (First Base, Seri Kembangan, Malaysia), 100 ng of genomic DNA and deionized water. Amplification was performed in a Biometra® TProfessional thermocycler with an initial 3 min of predenaturation at 95°C, followed by 35 cycles of denaturation at 95°C for 30 s, an optimum annealing temperature (as shown in table 2) for 30 s, 72°C for 30 s and a final extension step at 72°C for 5 min. The PCR products were separated by electrophoresis on 4% Metaphor agarose gel (Cambrex, Rockland, USA) in 1× TBE running buffer. In addition, 8% nondenaturing polyacrylamide gel was also used whenever the bands were unable to be discriminated distinctly in Metaphor (Rockland, USA) agarose gel. Both types of gel were stained with ethidium bromide and a 20 bp extended DNA ladder (Cambrex, Rockland, USA) was used as a size standard. The amplified bands were scored using the AlphaEaseFC™ version 4.0 software (Alpha Innotech Corporation, San Leandro, USA).

PopGene version 1.31 (Yeh *et al.* 1997) was used to calculate the observed and expected heterozygosities, while GenePop version 4.0.10 (Rousset 2008) was used to test for Hardy–Weinberg equilibrium (HWE) and linkage disequilibrium (LD). The number of alleles per locus ranged from 2 to 6 with an average of 2.8 alleles per locus (table 2). Locus PLlr1-9-2 showed the highest number of alleles per locus (6 alleles) followed by locus PLbp2-1-2 (5 alleles). The observed heterozygosities ranged from 0.000 to 1.000 (PLram1-2-1; Pantai Balok) and the expected heterozygosities ranged from 0.000 to 0.679 (PLbp13-5-5; Port Dickson) as shown in table 3. Overall in five populations, the observed heterozygosity was less than the expected heterozygosity. The mean of the observed and expected heterozygosities ranged from 0.259 (Pantai Bersih) to 0.364 (Pantai Balok) and from 0.346 (Sungai Muar) to 0.409 (Port Dickson), respectively. All 18 loci in each population were tested for conformity to HWE following a Markov Chain Method (table 3). 32 of 90 tests (35.6%) conformed to HWE ($P > 0.05$). The reasons accounting for the deviations from HWE may be the small sample size, inbreeding or the presence of null alleles. Seven pairwise comparisons of loci (PLbp13-5-5 and PLbp1-3-2, PLbp13-5-5 and PLbp13-5-3, PLbp13-5-5 and PLbp13-5-8, PLbp13-5-3 and PLbp13-5-7, PLbp11-10-2 and PLbp13-10-5, PLbp13-10-1 and PLbp13-10-6, PLbp13-10-5 and PLlr1-9-2) showed significant LD ($P < 0.05$) across all populations.

In conclusion, the 5′ anchored PCR technique proved to be a useful method for the isolation of microsatellite loci from *T. gigas* in a short time. This is the first report on microsatellite marker development and characterization for *T. gigas*, thus these markers will be useful to assess genetic variation for this species.

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