Conserv Genet (2009) 10:1807–1810
DOI 10.1007/s10592-009-9821-4

TECHNICAL NOTE

Isolation of polymorphic microsatellite loci from the endangered mudflat crab *Chasmagnathus convexus* (Crustacea: Decapoda: Varunidae)

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Received: 31 December 2008 / Accepted: 19 January 2009 / Published online: 5 February 2009

Abstract *Chasmagnathus convexus* is a mudflat crab that is distributed throughout East Asia. However, most populations of this species in Korea are endangered due to continuing seashore developments and habitat fragmentations. In this study, we isolated and characterized nine microsatellite loci from *C. convexus*. The number of alleles per locus ranged from 3 to 23. The expected heterozygosities ranged from 0.145 to 0.974 and the observed heterozygosities ranged from 0.150 to 0.947. Cross-species amplifications for 10 other Varunid crab species were performed. These microsatellite markers could be useful for planning conservation strategies for *C. convexus* and related species.

Keywords *Chasmagnathus convexus* · Microsatellite markers · Conservation · Cross-species amplification · Korea

Mudflat Crabs, *Chasmagnathus convexus*, live in intertidal and supratidal zones especially in estuary wetlands with reeds. This species is endemic to East Asia (Korea, China, Japan, Taiwan and northern Vietnam) (Shih and Suzuki 2008). However, most populations of this species in Korea are endangered due to continuing developments of river sides and seashores and habitat fragmentations. Therefore, this species has been designated as endangered by the Ministry of Environment, Republic of Korea. For its conservation and restoration, information about population structure is required. This can be indirectly obtained by molecular approaches using appropriate molecular markers such as microsatellite markers. Here we report nine microsatellite loci developed from *C. convexus* using enrichment methods.

Genomic DNA was extracted from ethanol-preserved specimens using Qiagen DNeasy Blood and Tissue kit (Qiagen). Approximately 5 μg of genomic DNA was restricted using BfuCI enzyme (New England Biolab). Digested DNA fragments greater than 400 bp were selected using Chroma spin column 400-TE (Clonetech laboratory). After SAULA/B linkers (Hammond et al. 1998) were ligated with the size- selected fragments using T4 DNA ligase (Promega), polymerase chain reaction (PCR) was performed using SAULA as a primer. The PCR mixture (total 25 μl) comprised 2 μl of linker-ligated DNA, 10 μl SAULA (10 μM), 1.5 μl of dNTP solution (10 mM), 5 μl 5× PCR buffer, 0.5 μl go Taq DNA polymerase (Promega), and 6 μl of distilled water (DW). PCR amplification was performed using GeneAmp 9700 (ABI).

The PCR was performed as follows: initial elongation at 72°C for 2 min, initial denaturation at 95°C for 3 min, 25 cycles of denaturation at 95°C for 3 min, annealing at 68°C for 1 min, elongation at 72°C for 1 min, and final elongation at 72°C for 10 min. For enrichments, the PCR products were hybridized with 3′-biotinylated probes [$(CA)_{11}$, $(GA)_{11}$, $(CAG)_{15}$, $(AAT)_{15}$, and $(CCT)_{15}$] in 6× SSC (1 M NaCl, 0.1 M NaCitrate). The mixtures were hybridized as follows: initial denaturation at 95°C for 5 min, then rapid ramps up to 70°C and steps aside of 0.2°C every 5 s for 99 cycles, and stationary at 50°C for 10 min, then steps aside of 0.5°C every 5 s for 20 cycles,

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Table 1 Characterization of 9 microsatellite loci from *Chasmagnathus convexus*

Locus	Primer sequences (5′-3′)	T_a (°C)	Repeat	Number of alleles	Size range (bp)	H_O	H_E	GenBank accession number
CC250	F: FAM-CTAGCCATCCCTTGGGAAAT	56	$(CA)_{38}$	18	133–187	0.8947	0.9331	FJ554584
	R: GAGCCAAGCCTGATAACCAC							
CC1006	F: FAM-GAGCAATGGTTAATGTGAAACAG	55	$(GT)_{36}$	21	104–186	0.9474	0.9218	FJ554576
	R: TGGTAATGTACGGAATATTGTGG							
CC1007	F: HEX-GGAAGGAGACGAAAGGAGGA	56	$(CCT)_7$	3	94–106	0.5790	0.4367	FJ554577
	R: CCGGAGTAAAAAGGCAGACA							
CC1017	F: NED-CTACCTCGCCTTCTCCTTCC	56	$(CCT)_{16}$	3	177–189	0.1500	0.1449	FJ554578
	R: CAATTTGCATATTTATGAAGTCGT							
CC1028	F: FAM-GGAAAAACAAACAAAGACTGAACA	57	$(CA)_{37}$	16	159–203	0.7647	0.9483	FJ554579
	R: TTGCTTTTAAGTGGGGAACG							
CC1029	F: HEX-TTGTGGGAGGAAGAGAGCTG	54	$(GGA)_{11}$	10	125–149	0.8421*	0.8777	FJ554580
	R: CTTGCCTCCTGCGTCTTG							
CC1031	F: NED-GCCGGACAAACAGACGAT	58	$(GGA)_{17}$	9	144–168	0.2500*	0.8949	FJ554581
	R: TTAATTCATTTTCCTGTGTTCTCTTG							
CC1034	F: FAM-TTGCAGTGTCTGTGCCTTTG	56	$(CCT)_8$	8	127–151	0.6500	0.8718	FJ554582
	R: CACACAGGTAGACCCTCAGGA							
CC1042	F: HEX-CCCGATGGCAAGTATGTTTT	55	$(GA)_{33}$	23	140–204	0.9474	0.9744	FJ554583
	R: GGGAGCGAGAGTAGATGTGG							

T_a annealing temperature, H_O observed heterozygosity, H_E expected heterozygosity

*significant departure from Hardy–Weinberg equilibrium ($P < 0.05$)

and finally rapid ramps down to 15°C. Biotin-hybridized DNA was captured using streptavidin-coated magnetic beads (Dynabeads M-280, Invitrogen) at room temperature for 15 min. Before capturing, magnetic beads were incubated for 10 min in 1.4× SSC and in 2× SPEP. The incubated beads were washed 4–5 times in 6× SSC, mixed with the biotin- hybridized DNA at room temperature for 15 min on a rotator, washed in 2× SSC and in 1× SSC. Finally DW was added to the beads and incubated at 95°C for 5 min, and the supernatant was quickly recovered.

Enriched DNA was amplified using SAULA as primer. After PCR, the products were purified using QIAquick PCR Purification Kit (Qiagen), after which DNA templates were cloned into p-Gem T-easy vector (Promega) according to the manufacturer's instructions. Positive clones were selected and incubated overnight at 36°C. Plasmid DNA was recovered using QIAprep Spin Miniprep Kit (Qiagen). Each colony was sequenced using BigDye Terminator Ready Reaction Mix (Applied Biosystems) with universal M13 primers. The sequences were determined using Genetic Analyzer 3770 (Applied Biosystems) and visually confirmed using Sequence Navigator 1.1 software (Perkin Elmer). The PCR primers for amplifications of the microsatellite regions were designed using Primer 3 software (Rozen and Skaletsky 2000). The forward primer was labeled with a fluorescent dye, such as FAM, HEX or NED.

Variability of these loci was screened using the DNA from 20 individuals that had been collected from four wild populations in Korea. Amplification was performed in 25 μl of a reaction mixture comprising 1 μl of genomic DNA, 0.5 μl of each primer (10 μM), 1 μl of dNTP solution (10 mM), 5 μl of 5× PCR buffer, 0.5 μl of go Taq DNA polymerase (Promega), and 16.8 μl of distilled water (DW). The PCR conditions were as follows: initial denaturation at 95°C for 5 min, 38 cycles of denaturation at 95°C for 30 s, annealing at optimal temperature (see Table 1) for 50 s, elongation at 72°C for 50 s, and final extension at 72°C for 30 min.

Tests for linkage disequilibrium and Hardy–Weinberg equilibrium used GENEPOP version 3.4 (http://wbiomed.curtin.edu.au/genepop/) (Raymond and Rousset 1995). This confirmed that there was no significant linkage disequilibrium between loci. Observed (H_O) and expected heterozygosities (H_E) were estimated using ARLEQUIN version 3.11 (Excoffier et al. 2005). As shown in Table 1, the number of alleles per locus ranged from 3 to 23. The expected heterozygosities ranged from 0.145 to 0.974, and the observed heterozygosities ranged from 0.150 to 0.974. CC1029 and CC1031 showed significant deviations from Hardy–Weinberg equilibrium.

Cross-species amplifications for the nine *C. convexus* loci were also conducted for 10 other species that belong to the same family Varunidae (Table 2): *Eriocheir sinensis*,

Table 2 Results of cross-species amplification with primer pairs developed from *Chasmagnathus convexus*

Species	Loci									
	CC250	CC1006	CC1007	CC1017	CC1028	CC1029	CC1031	CC1034	CC1042
Eriocheir sinensis	+	+	−	−	−	−	−	+	−
Cyclograpsus intermedius	−	+	−	−	−	−	−	−	−
Gaetice depressus	−	−	−	−	−	−	−	−	−
Hemigrapsus sanguineus	+	−	−	−	−	−	−	−	−
Hemigrapsus penicillatus	+	−	−	−	−	−	−	+	−
Helice tientsinensis	+	−	−	−	−	−	−	−	−
Helicana japonica	−	−	−	−	−	−	−	−	−
Helice tridens	+	−	−	−	−	−	−	−	−
Helicana wuana	+	−	−	−	−	−	−	−	−
Pseudohelice subquadrata	+	−	−	−	−	−	−	−	−

Two individuals of each species were screened. +, amplification; −, no amplification

Cyclograpsus intermedius, *Gaetice depressus*, *Hemigrapsus sanguineus*, *Hemigrapsus penicillatus*, *Helice tientsinensis*, *Helice japonica*, *Helice tridens*, *Helice wuana*, and *Pseudohelice subquadrata*. Results showed that the 3 loci were amplified in related species.

Acknowledgments This project was supported by a grant from the Eco-Technopia 21 Project funded by the Ministry of Environment, Korea and a grant from the Marine Biotechnology Programme funded by the Ministry of Land, Transport and Maritime Affairs of Korean government.

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