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# Isolation and characteristics of 10 microsatellite markers from the endangered coconut crab (*Birgus latro*)

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## Abstract

**The coconut crab (*Birgus latro*), an endangered marine-dispersed crustacean, is facing severe and probably accelerating population extinction worldwide, but biological information on its conservation remains deficient. In order to reveal the genetic structure of *B. latro*, 10 microsatellite loci were developed. A high degree of polymorphism was observed with a mean number of alleles per locus of 16.9. The mean expected heterozygosities were also high, ranging from 0.742 to 0.965. The observed heterozygosities ranged from 0.210 to 0.925. Departures from Hardy–Weinberg equilibrium were observed at five loci after the Bonferroni correction. These hypervariable markers will be utilized to study the genetic diversity and conservation of *B. latro* throughout its distribution range in the Pacific and Indian Oceans.**

*Keywords*: *Birgus latro*, coconut crab, Coenobitidae, endangered species, microsatellites

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The coconut crab *Birgus latro* (L.) is a monospecific genus in the Coenobitidae with a widespread distribution on isolated tropical islands throughout the Pacific and Indian Oceans. However, the coconut crab is facing severe conservation problems nowadays. Populations on most inhabited islands have been severely depleted or extirpated entirely due to human interference (Fletcher *et al*. 1990; Wang *et al*. 2007). The coconut crab has been listed on the International Union for the Conservation of Nature (IUCN) Red List since 1983 (Wells *et al*. 1983), and is also the only crustacean species protected under the Wildlife Conservation Law in Taiwan since 1995. According to the IUCN Red List criteria, there is insufficient information on this species to make an assessment of its risk of extinction based on its distribution and/or population status (www.iucnredlist.org). Information on the genetic structure and demographics is needed to evaluate gene flow and identify population units of this endangered species.

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Lavery *et al*. (1996) utilized mitochondrial DNA (mtDNA) restriction fragment length polymorphism (RFLP) to examine the population genetics of *B. latro* collected from the Pacific Ocean and Christmas Island of the Indian Ocean. The results revealed the island populations diverged in the pattern of isolation by distance in the Pacific and Indian Oceans, and island populations varied under a spatial scale consideration, which was also observed in an allozyme study (Lavery *et al*. 1995). However, allozyme and mtDNA RFLPs are limited in resolving fine-scale differentiation and subdivision patterns among populations. In addition, there is no genetic information available for isolated remnant populations, such as those of Chagos, Zanzibar, and the Aldabra atoll in the West Indian Ocean. In a joint effort of an international consortium to determine the conservation genetics of *B. latro*, alternative nuclear markers, hypervariable microsatellite loci, were developed to resolve the small scale of gene flow, genetic subdivisions, and connectivity of currently existing populations.

Microsatellite loci were isolated from specimens sampled from Green Island, off the southeastern coast of Taiwan

**Table 1** Characteristics of microsatellite markers isolated from *Birgus latro*

| GenBank Accession no. | Locus | Repeat motif | Primer sequence (5′–3′) | $T_a$(°C) | Size range (bp) | No. of alleles | $H_O$ | $H_E$ | *P* value (HWE) |
|---|---|---|---|---|---|---|---|---|---|
| EU789581 | GBL01 | $(GA)_{27}$ | F: TTGAGACAAATAGTGTGTGCATTG<br>R: AGCCACAATATCAGGGCACAAG | 60 | 153–193 | 14 | 0.259 | 0.921 | $P < 0.0001$* |
| EU789582 | GBL02 | $(GA)_{28}$ | F: GGGTGAGGTAAAGGCTGCTGTG<br>R: ACACTTAAAATGTTTGGCAGG | 58 | 104–202 | 24 | 0.680 | 0.961 | 0.00024 |
| EU789583 | GBL03 | $(GA)_{31}$ | F: TGGTGTTTGAATTTGCATAACG<br>R: GATGATGGGAAGCGACGAGG | 60 | 80–140 | 6 | 0.210 | 0.711 | $P < 0.0001$* |
| EU789584 | GBL04 | $(GA)_{17}$ | F: CTGATTGGAGTAGGGAGGATGG<br>R: TGCATTAATTGTGAGGTTTACCAGC | 60 | 214–279 | 17 | 0.925 | 0.897 | 0.10727 |
| EU789585 | GBL05 | $(TA)_{19}$ | F: GGCAAAGTTCCCAGTCTGCTG<br>R: AAGCCATGGAAGTGCAACGAACC | 60 | 210–314 | 18 | 0.312 | 0.965 | $P < 0.0001$* |
| EU789586 | GBL06 | $(GT)_{34}$ | F: CCCTCCACCGCCTGTTGC<br>R: AAAAGAAGGTGACGAGGCTCTG | 60 | 178–224 | 12 | 0.730 | 0.742 | 0.05688 |
| EU789587 | GBL07 | $(GT)_{38}$ | F: TTGCATGTCTTGTGCCCTG<br>R: TTTAATTCGTTCGGTCAGG | 58 | 114–174 | 16 | 0.684 | 0.943 | 0.00576 |
| EU789590 | GBL08 | $(GA)_{16}G(GA)_1$ | F: GTGGATGCAGAGCCGTAGTCC<br>R: TGTGGAAGACTCGTTTCCTCG | 62 | 140–186 | 17 | 0.222 | 0.935 | $P < 0.0001$* |
| EU789589 | GBL09 | $(TG)_{29}CG(TG)_{28}$ | F: GATCCTGACCGACCCGG<br>R: TCTTCCTCATTGCCAAGGTCG | 60 | 136–294 | 26 | 0.851 | 0.912 | $P < 0.0001$* |
| EU789588 | GBL10 | $(GTA)_{10}TTA$ $(GTA)_4.(TG)_{63}$ | F: GGTTCAAGGACAGTGGGGATGC<br>R: TCGTATTTGCTTCTGCTTTCGAG | 60 | 131–295 | 19 | 0.615 | 0.892 | 0.00043 |

$H_O$ indicates observed heterozygosity.
$H_E$ indicates expected heterozygosity.
*indicates significant deviation from Hardy-Weinberg equilibrium (HWE) after Bonferroni correction ($P < 0.0001$).

(22°41′16.82″N, 121°27′45.03″E) in August 2005. A genomic DNA library of *B. latro* was constructed using standard molecular protocols (Sambrook *et al*. 1989) with some modifications. Total genomic DNA was extracted from 90% ethanol-preserved muscle tissue using a phenol/chloroform method (Sambrook & David 2001). Approximately 50 ng/μL DNA was separately digested with blunt-end restriction enzymes of *Rsa*I, *Alu*I, *Hae*III, and *Hin*cII (Biolabs). Digests were run on 2% Nusieve agarose gels (FMC Bioproducts) and stained with ethidium bromide. Fragments of between 300 bp and 1000 bp were excised from the gel and purified using a phenol/chloroform extraction. Selected fragments were ligated into a *Sma*I-digested pUC18 vector (Amersham), transformed into *Escherichia coli* DH5α-competent cells (Invitrogen), and plated onto LB/ampicillin/IPTG/X-gal agar plates. Recombinant colonies were identified using blue/white selection and lifted onto Hybond-N+ nylon membranes (Amersham). DNA from these colonies was cross-linked with the membrane and hybridized with the oligonucleotide probes $(AT)_{10}$, $(GA)_{10}$, and $(CG)_{10}$ following the manufacturer's instructions of the ECL nucleotide labelling and detection kit (Amersham-Pharmacia). A second screening was conducted to confirm positives colonies.

In total, 46 positive clones were obtained out of 230 white colonies. The insert lengths of these positive colonies were determined using standard forward and reverse M13 primers, and then the polymerase chain reaction (PCR) products sized between 400 bp and 1000 bp were sequenced. Ten primer pairs were designed according to a highly conserved flanking region of microsatellite motifs using the software Oligos 9.9 (Primer Digital). All of these were dinucleotide repeats: seven were perfect repeats, two imperfect repeats, and one a compound repeat (Table 1).

To characterize the isolated microsatellites, 27 coconut crabs samples were collected from Lanyu (Orchid Island), off the southeastern coast of Taiwan (22°02′01.05″N, 121°32′18.20″E). The tibia of the fourth leg of 11 male and 16 female coconut crabs were collected, and individuals were then tagged and released. Total DNA of *B. latro* was extracted from fourth tibia using (cetyltrimethyl ammonium bromide) CTAB protocol described by Ausubel *et al*. (1995) with the following modification: all centrifugation was carried out at 1400 ***g***, the DNA pellets were washed in 200 μL washing buffer (76% ethanol and 10 mM ammonium acetate), and the dried DNA pellets were redissolved in 50 μL TE buffer [10 mM Tris-HCl (pH 7.4) and 1 mM EDTA]. Samples were treated with 0.03 μg/mL RNase A (Ribonuclease, Viogene) and incubated at 37 °C for 2 h. PCR was performed in 10-μL volumes, containing 0.5 U of FastStart *Taq* (Roche), 1× PCR buffer, 0.2 mM dNTP each, 0.2 μM forward HEX-labelled primer and reverse unlabelled primer,

and about 0.01 g template DNA. The PCR was performed in PXE thermal cyclers (Thermo Electron). PCR cycling conditions were 94 °C for 5 min, followed by 35 cycles of 94 °C for 30 s, the optimal annealing temperature for 30 s (Table 1), 72 °C for 30 s, and a single extension at 72 °C for 5 min. Following the PCR, and two volumes of bromophenol blue in deionized formamide solution was added to each sample, denatured for 10 min at 95 °C, and electrophoresed on 5% denaturing polyacrylamide gels using the GeneScan 3000 (Corbett Robotics). Alleles were determined according to TAMRA-500 size markers (Applied Biosystems) with the Gene Profiler 4.05 (Scanalytics). The potential presence of null alleles or errors caused by large allele drop-out or stuttering were assessed using MICRO-CHECKER 2.2.3 (van Oosterhout *et al*. 2004). Number of alleles per locus, expected and observed heterozygosities, and exacttests of linkage disequilibrium and Hardy-Weinberg equilibrium (HWE) were calculated using MSA software (Dieringer & Schlötterer 2002) and ARLEQUIN version 3.1 (Excoffier & Schneider 2005). Alternative hypotheses of a heterozygote excess or deficiency were tested using GenePop DOS version 3.4 (Raymond & Rousset 1997).

Ten loci were polymorphic among the 27 coconut crabs from Lanyu, Taiwan. The mean number of alleles per locus was 16.9. At least 12 alleles could be amplified at all loci except GBL03 wherein only six alleles could be amplified (Table 1). Ten out of 45 comparison loci (GBL01/GBL05, GBL08, GBL09, GBL02/GBL09, GBL03/GBL05, GBL08, GBL05/GBL08, GBL09, GBL10, GBL08/GBL10) presented linkage disequilibrium ($P < 0.0001$) after the Bonferroni correction (Rice 1989). The mean observed and expected heterozygosities were calculated as 0.549 (range, 0.210–0.925) and 0.888 (range, 0.742–0.965), respectively. Five loci showed HWE deviations ($P < 0.0001$) following Bonferroni correction, and heterozygote deficits were shown at loci GBL04, GBL06, and GBL07. Potential occurrences of null alleles were detected at loci GBL01, GBL02, GBL03, GBL05, GBL07, GBL08, GBL10 with frequency ranging from 0.023 to 0.713, and might have resulted in heterozygote deficits. We are currently evaluating the utility of these markers to resolve the population genetics of *B. latro* in its distributional range throughout the Pacific and Indian Oceans. The results will be utilized to answer questions of genetic connectivity of coconut crabs at different spatial scales and to draw implications for their conservation.

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