Development and Characterization of 20 Microsatellite Markers for Chinese Black Sleeper, *Bostrychus sinensis*

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Received: 12 October 2011; in revised form: 30 November 2011 / Accepted: 12 December 2011 / Published:

**Abstract:** Twenty microsatellite markers were isolated and characterized from the Chinese black sleeper, *Bostrychus sinensis*. Loci were screened in 30 individuals from Taiwan. For each locus, the number of alleles varied from 4 to 22 with mean expected and observed heterozygosity of 0.79 and 0.66, respectively. One locus significantly deviated from Hardy-Weinberg equilibrium after Bonferroni correction and no significant linkage disequilibrium was detected. This set of microsatellites will provide a suitable tool for population genetic studies of Chinese black sleeper.

**Keywords:** *Bostrychus sinensis*; microsatellites; population genetic
1. Introduction

The Chinese black sleeper, *Bostrychus sinensis* (Lacepede 1801), is one of the most widespread species of Indo-Pacific eleotrids, manifesting on the northern Indian Ocean coast, reaching east to the Pacific, Melanesia and Polynesia, north to Japan, and south to Australia [1,2]. In China, it is distributed in the coastal area of the East China Sea, Taiwan Strait and South China Sea. Chinese black sleepers are burrowing amphibians. They live in a limited territory, spawn in their burrow, and exhibit egg-guarding behavior. These factors suggest that populations will be sensitive to local environmental conditions and have a low rate of dispersal. Therefore, *Bostrychus sinensis* can be a useful biological indicator of the effects of long-term historical vicariant events and short-term human activities on intertidal habitats. In order to facilitate its population genetic studies, we developed and characterized 20 polymorphic microsatellite markers from Chinese black sleeper.

2. Results and Discussion

The number of observed alleles per locus ranged from 4 to 22. The observed and expected heterozygosity values ranged from 0.200 to 0.889 and 0.186 to 0.933, respectively. One locus (S98W83) significantly deviated from Hardy-Weinberg equilibrium after Bonferro correction ($P < 0.0025$) and no significant genotypic linkage disequilibrium (LD) was found between all pairs of these 20 loci after Bonferroni correction ($P > 0.0025$). The levels of polymorphism uncovered at these loci suggest that they should be useful for population genetics as well as phylogeographic studies.

3. Experimental Section

3.1. Isolation of Microsatellite Markers

Microsatellites from *B.sinensis* were isolated using a modified enrichment technique described by Ding [3]. Genomic DNA was extracted from the muscle tissue using a standard traditional phenol-chloroform procedure [4] and digested with *Sau*3AI at 37 °C overnight. Fragments from 500–2000 bp were excised from agarose gels using a QIAquick Gel Extraction Kit (QIAGEN) and ligated to two oligo adapters (Oligo A 5′-GATCGTCGACGGTACCGAATTCT-3′ and Oligo B 5′-GTCAAGAATTCGGTACCGTCGAC-3′) to facilitate amplification by PCR. The amplified genomic fragments were subsequently hybridized with the 5′- biotinylated oligo probes ATA(CA)22C and fragments containing potential repeat motifs were captured with steptavidin-coated magnetic beads (Dynabeads® M-280, Invitrogen). To increase the amount of potential repeat motifs, a “recovery” PCR was performed using oligo B as the PCR primer. The PCR products were then purified and ligated into PMD19-T vector (TAKARA) and transformed into DH5α competent cells. Cells were then plated onto LB agar, X-gal and ampicillin and incubated overnight at 37 °C.
Table 1. Details for 20 polymorphic microsatellite loci developed for *Bostrychus sinensis*.

| Locus Genbank no. | Repeat motif | Primer sequence (5’-3’) | T_a (°C) | Size range (bp) | Na | H_o/H_e | P-value |
|------------------|--------------|-------------------------|---------|-----------------|----|---------|---------|
| BSD026           | (CA)_61      | F: CATAAAAGACCCATTGTAACCTGCT  | 58      | 256–280          | 8  | 0.865/0.825 | 0.0505  |
| JN806116         |              | R: CTGTAGGCCCTCAGGAGCACAATA |         |                 |    |         |         |
| BSD121           | (AC)_19      | F: CGCACTGTGTCATGATGACTC  | 58      | 131–175          | 8  | 0.444/0.760 | 0.0060  |
| JN806117         |              | R: CCACCTGAACATGTTAGTT    |         |                 |    |         |         |
| BSD137           | (TG)_23      | F: CTGACCTGGACTCCTCCTG    | 58      | 202–330          | 22 | 0.879/0.933 | 1.0000  |
| JN806118         |              | R: CTGGGACAGGAGTGGATT     |         |                 |    |         |         |
| BSB006           | (TG)_6 C(GT)_13 | F: TATTCCTGTAATATCAGATGCTGCA | 60      | 172–222          | 9  | 0.742/0.843 | 0.2209  |
| JN806119         |              | R: TACACAAGACAAAAAGTTAGGAA |         |                 |    |         |         |
| BSW045           | (CT)_6 ...(AC)_70 | F: AACTTTTTTTCTCAATTGTTGCTTAA | 52      | 128–220          | 14 | 0.833/0.867 | 0.9975  |
| JN806120         |              | R: TGTGCTCAGGGTACCGGGA    |         |                 |    |         |         |
| BSW068           | (AC)_16      | F: CTACACAGCAGCAGCAACC    | 58      | 113–133          | 7  | 0.684/0.766 | 0.3483  |
| JN806121         |              | R: ACTCCAAACACTGCTCAAGAAC |         |                 |    |         |         |
| BSSD14           | (TG)_43      | F: ATTTAGCAGGCTTTATTTT    | 55      | 200–244          | 11 | 0.567/0.754 | 0.5198  |
| JN806122         |              | R: GGCCTGCTTCCATCTTTTCT  |         |                 |    |         |         |
| BSSD21           | (TG)_38      | F: GATCCATCTTAAACACTCGTTAT | 55      | 267–313          | 9  | 0.774/0.833 | 0.6818  |
| JN806123         |              | R: CAGGAGCAGTACACAGACAAAA |         |                 |    |         |         |
| BSSW83           | (TG)_16      | F: CACGCGACGCTGACACTCCAT  | 58      | 144–156          | 8  | 0.452/0.808 | 0.0000 *|
| JN806124         |              | R: TCCAGTGTTTGAAACTCCTGCC |         |                 |    |         |         |
| BSE020           | (GT)_25      | F: GATTTTACAGGACAGCGCTTTGCC | 66      | 239–339          | 10 | 0.769/0.824 | 0.9217  |
| JN806125         |              | R: CCACAAAACGGAGGCTCCCAATCT |         |                 |    |         |         |
| BSSW87           | (GT)_35      | F: CGCAGATGTGCGCTCCTTTTTA | 64      | 314–388          | 10 | 0.680/0.860 | 0.7399  |
| JN806126         |              | R: GCCTCGCTGCTCCTCCTCCT  |         |                 |    |         |         |
| BSSW89           | (GT)_33      | F: TTTGAGCATCTTCTGCTGCTTG | 52      | 182–246          | 10 | 0.583/0.884 | 0.1589  |
| JN806127         |              | R: CTGACTCCATCGGAATGTGCTTA |         |                 |    |         |         |
| BSD106           | (CA)_20 T(AC)_9 | F: GAGATGAGCACAAGGGTGGAGTC | 56      | 338–388          | 10 | 0.667/0.842 | 0.5932  |
| JN806128         |              | R: CTGGCAGAAAGGGATTGAGG   |         |                 |    |         |         |
| BSD045           | (GT)_35      | F: AAATGGAATGTGAGAAGATGTGAGGCA | 62      | 258–384          | 10 | 0.467/0.720 | 0.4404  |
Table 1. Cont.

| Locus Genbank no. | Repeat motif | Primer sequence (5′-3′) | $T_a$ (°C) | Size range (bp) | Na | $H_o$/$H_e$ | $P$-value |
|------------------|--------------|-------------------------|----------|----------------|----|------------|-----------|
| BSW115           | (GT)25C(TG)17| F: TGTGATGTGTGTTTTGGGTGGTTA R: TGTGTCTCTGAAGTGCTGAAGC | 64       | 437–541        | 7  | 0.708/0.764| 0.8613    |
| JN806130         |              | F: TGGGCTCAGTTCTGTGGAGGTA R: CGAGGATGAGGCAGGCTAGGACT | 66       | 308–394        | 17 | 0.889/0.912| 0.9997    |
| BSW053           | (AC)5…(CA)7…(AC)50 | F: GCCCCGCTACCCGACATTA R: CGAGGATGAGGCAGGCTAGGACT | 56       | 137–185        | 9  | 0.714/0.827| 0.9570    |
| JN806131         |              | F: CGCTTCAAGTTCTGTGGAGGTA R: CGAGGATGAGGCAGGCTAGGACT | 56       | 137–185        | 9  | 0.714/0.827| 0.9570    |
| BSD125           | (CA)2CGCACG(CA)35 | F: CGGTTCAAGTTCTGTGGAGGTA R: CTGTCTGCTGGCTGTGGTA | 56       | 137–185        | 9  | 0.714/0.827| 0.9570    |
| JN806132         |              | F: CGCTTCAAGTTCTGTGGAGGTA R: CGAGGATGAGGCAGGCTAGGACT | 56       | 137–185        | 9  | 0.714/0.827| 0.9570    |
| BSC001           | (GT)48       | F: CTTGTTAGTGTTAAACCCGTAAGCTTTA R: CCTATGTCGCTTGGGCTAGGACCG | 56       | 341–401        | 15 | 0.615/0.909| 0.4762    |
| JN806133         |              | F: CTTGTTAGTGTTAAACCCGTAAGCTTTA R: CCTATGTCGCTTGGGCTAGGACCG | 56       | 341–401        | 15 | 0.615/0.909| 0.4762    |
| BSE008           | (TG)19       | F: GCTGCTCATACAAATAATCTCTC R: GTTGTCTGTAATCGTGGCTCTA | 58       | 138–154        | 6  | 0.656/0.734| 0.7305    |
| JN806134         |              | F: GCTGCTCATACAAATAATCTCTC R: GTTGTCTGTAATCGTGGCTCTA | 58       | 138–154        | 6  | 0.656/0.734| 0.7305    |
| BSC002           | (ACT)18      | F: ATCGAGACTCAATGACCTGGGAG R: CTGGTTGAACAGCTACTTC | 55       | 247–271        | 4  | 0.200/0.186| 0.9926    |
| JN806135         |              | F: ATCGAGACTCAATGACCTGGGAG R: CTGGTTGAACAGCTACTTC | 55       | 247–271        | 4  | 0.200/0.186| 0.9926    |

$T_a$: Annealing temperature (°C), $Na$: Number of alleles, $H_o/H_e$: Observed heterozygosity and Expected heterozygosity, $P$-value: $P$-values for exact tests for Hardy-Weinberg equilibrium (HWE), * show significant deviation from HWE after Bonferroni correction ($P < 0.0025$)
The positive clones were identified by PCR with vector-specific primers. After being identified, 196 positive clones were randomly selected for sequencing using ABI3730XL sequencer (Applied Biosystems). Chromatograms were assembled and edited using SEQUENCER 4.9 (Gene Codes Corporation), and 70 primers were designed for each unique amplicon containing a target microsatellite repeat. Initially, eight samples from different localities were used to test amplification of loci and evaluate polymorphic content. The PCR amplification was performed in 15 μL volume containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl₂, 0.2 mM each dNTPs, 0.4 μM each primer, 1 U Taq polymerase (TAKARA) and 30 ng genomic DNA. After denaturation for 5 min at 94 °C, followed by amplification for 30 cycles (94 °C for 30 s, annealing temperature for each pair of primers (Table 1) for 30 s, 72 °C for 30 s) and a final step at 72 °C for 5 min. PCR products were mixed with the GS500LIZ size standard (Applied Biosystems) and formamide and run on an ABI3130xl DNA Analyzer. Fragment analysis and genotyping were performed using Genemapper version 4.0 (Applied Biosystems). Out of the 70 primer pairs tested, 20 pairs were successfully amplified by PCR and further characterized using additional samples at Chiku Lagoon (23°55'05" N-120°02'57" E) from Taiwan (n = 30).

3.2. Data Analysis

The number of alleles, observed and expected heterozygosities, P value of Hardy-Weinberg and linkage disequilibria were estimated by using POPGENE [5].

4. Conclusions

In the present study, we describe 20 polymorphic microsatellite loci shown as the first set of microsatellite markers designed specifically for Bostrychus sinensis. These loci would be useful in providing an effective tool for investigating genetic variation and population structure in B. sinensis. Microsatellites are an excellent choice of genetic marker for genome mapping due to their hyper-variability and abundance throughout most vertebrate genomes. In our results, there are some strong heterozygosities for microsatellite loci, like BSD137/BSW045/BSW053/BSC001, which are suitable for identifying genetic mapping in B.sinensis. These markers will prove helpful in the management of fisheries and in the design of conservation strategies.

Acknowledgments

This work was supported by Nature Science Foundation of China (Grant No. 40976094) and key programme of Science and Technology Department Foundation of Fujian Province (Grant No. 2011N0034.). We are grateful to Maoyong for the collection of samples.

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