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TECHNICAL NOTE

Isolation of microsatellite loci from the millipede, *Brachycybe nodulosa* Verhoeff

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Abstract *Brachycybe nodulosa* is a fungivorous millipede that distributed in Japan and Korea. This species shows patchy distributions and the exclusive paternal care of eggs, and thus is an important subject for both conservation and evolutionary biology. Thirteen polymorphic microsatellite loci were newly isolated from the taxon. Moderate to high levels of polymorphism were observed, with numbers of alleles ranged from 2 to 10. The mean observed and expected heterozygosities ranged from 0.200 to 1.000 and 0.405 to 0.886, respectively. Two loci showed lower heterozygosities than the expectation under HWE. No linkage disequilibrium was detected for any pair of the loci. These new loci will be useful in conservation genetics and evolutionary ecology studies of this species.

Keywords Microsatellite · Millipede · Genetics · Paternal care

The millipede genus *Brachycybe* (Andrognacidae) comprises seven species; five species are known from North America and two from East Asia (Shelley et al. 2005). *Brachycybe nodulosa* Verhoeff is a species found in central and southwestern Japan, and also recorded in the coastal tip of South Korea (Shelley et al. 2005). Distribution of this species seems to be restricted to mountain regions covered by forests of the Japanese cedar *Cryptomeria japonica*, which can lead to geographically fragmented and subdivided populations, sometimes in small size. This species is fungivorous and, in breeding seasons, usually colonizes on the underside of decaying logs (Murakami 1962; Kudo et al. 2011). Such microhabitat nature, in addition to their low vagility, may have resulted in populations genetically structured in fine-scale. Analyzing genetic variability within and among populations will be essential for conservation biology studies. On the other hand, interestingly in *B. nodulosa* (Murakami 1962; Kudo et al. 2011), as well as other *Brachycybe* spp. (Gardner 1975; Tallamy 2001) and related taxa (Kudo et al. 2009), males receive eggs from females and care the mass of eggs until hatching. Parentage of cared offspring is quite important to understand possible selection mechanisms for the evolution of exclusive paternal care (Tallamy 2000). For example, if low paternity and multiple-maternity would be detected in clutches cared by males, the sexual selection hypothesis, in which egg-caring males are favored due to female preference for those males as mates, would be supported (Tallamy 2000). Although suitable genetic markers, such as microsatellite loci, are required for those studies, it is available only for limited millipede taxa (Wojcieszek and Simmons 2009). Here, we report the characterization of thirteen microsatellite loci useful for conservation genetics and evolutionary ecology studies of *B. nodaulosa*.

Specimens of *B. nodulosa* were collected in the Shimabara peninsula, Kyushu, Japan. Millipedes were preserved and stored in 99.5% ethanol. Genomic DNA was isolated from an adult female using the DNeasy tissue Kit

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(Qiagen). Isolation of microsatellite loci was performed following a microsatellite enrichment method (Hamaguchi et al. 2007) using the magnetic beads coated by stepavidin (Promega). Digested DNA by Sau3AI was ligated to an adapter (Cassette Sau3AI, TaKaRa) with T4 DNA ligase (TOYOBO). The ligated DNA was enriched for a biotinylated ologonucleotide repeats $(GT)_{10}$ using the magnetic beads (Promega). Fragments were cloned using pGEM-T vector (Promega) and the XL1-Blue MRF' bacterial host strain (Stratagene). Inserted DNA of color-positive clones were amplified by PCR and screened for microsatellite regions by a membrane based dot blot using the detection kit for the biotinylated DNA probe (Imaging-High-Color, TOYOBO). Positive clones were checked for insert size by PCR using an MJ research PTC-100. The 25 µl reactions contained 1 µl template DNA, 0.2U of Ex-taq (TaKaRa), 0.8 µM each of primer, 2.5 µl of 10× PCR buffer (TaKaRa) and 200 µM of each dNTP. Clones that exhibited a single band of 400-1000 bp were cleaned using the DNA purification kit (Qiagen) and were sequenced using the DTCS quick start kit (Beckman-Coulter). Sequences were electrophoresed on a CEQ-8000 genetic analyzer (Beckman-Coulter).

Each of eighty-one inserts contained a microsatellite region with more than five repeat units, for which primers were designed. A DNA mix of 20 *B. nodulosa* individuals was amplified for each locus, and the products were separated by a polyacrilamide gel electrophoresis (Hasegawa and Imai 2004). Only loci showing multiple bands in this preliminary analysis were checked for allelic polymorphism using dyed primers with the Beckman dyes. Thirteen loci were found to be polymorphic, and the number of alleles per locus ranged from 2 to 10 in 20 individuals (Table 1). Observed and expected mean heterozygosities

Table 1 Primer sequence and diversity statistics for thirteen microsatellite loci isolated from *Brachycybe nodulosa*

Locus	GenBank accession number	Primer sequence (5′-3′)	Repeat motif	AT(°C)	Allele sizes range	Genetic diversity			
						N_A	H_O	H_E	HWE *P* value
ys1	AB615201	F-CCAGTGTATCCAGGTGCACAGTGGCAA	$(CA)_{10}$	55	145–147	4	0.600	0.610	0.278
	AB615202	R-CAACCGAGTAAGCACACGTAGACGGAA							
ys15	AB615203	F-GGTTTCAGATGGCATTGGTATTGCA	$(CA)_{12}$	55	248–258	6	0.778	0.790	0.164
	AB615204	R-CAACAACTGGCTCTTCTTTGCCCT							
ys619	AB615205	F-TTTAAGAGGTGACAATCCTCCTTGT	$(GT)_9$	55	202–208	3	0.750	0.664	0.391
	AB615206	R-GCCGGTGCGAGGCTCCGAATCTGGT							
ys683	AB615207	F-CTAAAGCGTGGAACACACTCGCGGA	$(CA)_8$	55	184–199	7	0.625	0.773	0.819
	AB615208	R-GTGGATAGGAGGGGCGGCTTGT							
ys701	AB615209	F-GGTATCTTGTAGTGGGTGGAGTT	$(GT)_{13}$	55	218–224	4	0.625	0.680	0.118
	AB615210	R-CGTACCGCTTTGAATAATTGCCGA							
ys817	AB615211	F-GCCACATTTATTATGTTAAATTCCT	$(GT)_9$	55	141–154	2	0.556	0.401	0.103
	AB615212	R-CTTATAAGATGTAATCGCGAGAGT							
ys836	AB615213	F-GCTTAATGCTCTTTTGGTACGACA	$(TG)_{10}$	55	154–158	4	0.200	0.635	0.002
	AB615214	R-GAATTCCTTAACGCAACGAGCGCAA							
ys871	AB615215	F-GTAGAAGCTCAATGGTATCACCACA	$(GT)_9$	52	195–203	4	0.625	0.539	0.524
	AB615216	R-CACGAGTCACGTAGACACAGTACGA							
ys881	AB615217	F-AATACATTCACAATTACAAACGCA	$(CA)_9$	55	148–168	8	0.900	0.835	0.130
	AB615218	R-CTTGTCATGTGTTTCACTCTGTCAT							
ys936	AB615219	F-ATATCTCACAATTATCGAAAGAACA	$(CT)_6(CA)_{16}$	55	197–243	10	1.000	0.886	0.297
	AB615220	R-GTGCATAGCTCATTAGTGGACGA							
ys937	AB615221	F-CAAATGGTATATTGATTGTGAGGTT	$(GT)_9$	52	156–160	3	0.364	0.460	0.067
	AB615222	R-AAGGGGAAGTTTACAACATCAAAGT							
ys949	AB615223	F-CAAATCAACCCGTCTCATGTGCCT	$(AT)_5(AC)_8$	55	140–142	2	0.125	0.305	0.019
	AB615224	R-GAGTTGTAGTGTAGAGTTGCCTA							
ys954	AB615225	F-GAACGGGCACTTCGGGCGGTAGTA	$(CA)_{17}$	55	140–144	3	0.500	0.405	0.000
	AB615226	R-GGAAGAAGTTTCTTGGACTTTGAGA							

Shown are loci name, the GenBank accession numbers, the forward (F) and reverse (R) primer sequence, repeat motif of the sequenced clone, temperature at annealing (AT), allele size range in base pairs, the number of alleles (N_A), observed heterozygosity (H_O), and expected heterozygosity (H_E) in the population (N = 20), and *P* value associated with departure from Hardy–Weinberg Equilibrium (HWE)

ranged from 0.200 to 1.000 and 0.405 to 0.886, respectively. Two loci, ys683 and ys949, showed significant deviations from HWE, and no loci exhibited significant linkage disequilibrium.

We conducted a cross-species amplification using 10 individuals of the related species, *Yamasinaium noduligerum*. Each locus was amplified by using the same cycle with *B. nodulosa* (see Table 1 for condition). However, no locus could amplify the target region.

The microsatellite loci described in this paper will be useful for investigating the population structure, levels of genetic variability and genetic differentiation among subdivided populations. Additionally, the markers are also valuable in parentage analysis of egg clutches brooded by males in the field.

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