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PRIMER NOTE

Characterization of 12 polymorphic microsatellite loci in the Japanese bush warbler *Cettia diphone*

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Abstract

Japanese bush warblers, *Cettia diphone*, are a common species in Japan and have a polygynous breeding system. In the breeding ground some males have their own territories with more than one female. Male floaters are also not uncommon in the breeding ground. To understand breeding strategy in this species, exact parentage should be elucidated. In order to obtain a tool for this purpose, we isolated 34 microsatellite loci from a genomic library in this species and developed primers for 12 loci. These primers were tested in the Japanese bush warbler and successfully amplified. In analyses of 49 unrelated individuals, allelic numbers ranged from two to 22, and observed heterozygosity (H_O) ranged from 0.27 to 0.854 except for two loci (Cdi29 and Cdi35a) with $H_O < 0.1$.

Keywords: *Cettia diphone*, Japanese bush warblers, microsatellite, primer

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Japanese bush warblers (*Cettia diphone*) are distributed along the eastern rim of the Eurasian continent and the Japanese islands and are one of the best known species in Japan because of their unmistakable songs (Hamao 1997). They are residents or short-distant migrants on Honshu (main) island in Japan. In early spring, males begin to sing in wintering sites, such as bushes, parks, and backyards of lowland areas and most of them migrate to higher mountainsides and breed in bamboo bushes or grass meadows. At the field site in Chichibu, Honshu island, we observed their behaviour and collected blood samples for hormone measurement after capture with mist nets. We found that males sing from late March through August in the breeding area and circulating testosterone is found high during this period (Wada *et al.* 1999). The bush warblers often take a polygynous breeding strategy if the resource is abundant. In the breeding ground some males have their own territories with more than one female. Male floaters are also not uncommon in the breeding ground. Territorial males occasionally change their territories during a breeding season. To understand breeding strategy in this species, exact parentage should be elucidated.

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As far as we know, no nuclear markers have been specifically developed for bush warblers. Nishiumi *et al.* (1996) have developed primers for great reed warblers (*Acrocephalus arundiceus*), but they did not show any positive result with bush warblers. Thus we decided to construct a genomic library and develop primers for the species. DNA for library construction was obtained from blood samples of birds captured in Chichibu. Genomic DNA was digested using Sau3AI. Digested DNA fragments ranging from 400 to 800 bp were ligated into pUC118BamHI/BAP (Takara) and transformed into *Escherichia coli* JM109 Electro-Cells (Takara) by electroporation. Colonies were transferred into positively charged nylon membrane (Biodyne B, Pall) and then hybridized with $(GT)_{15}$ $(GA)_{15}$ $(AAAC)_5$ $(AAAG)_5$, labelled with digoxygenin using DIG Oligonucleotide 3′-End Labeling Kit (Roche). Hybridized probes were detected using DIG Nucleic Acid Detection Kit (Roche). Out of approximately 10 000 colonies screened, we found 34 positive colonies, which were sequenced using BigDye Terminator Cycle Sequencing FS Ready Reaction Kit (Applied Biosystems) on an ABI 310. Out of 34, 12 clones contained a microsatellite sequence, and primers were designed for each locus.

Polymerase chain reaction (PCR) amplifications were performed using Takara PCR Thermal Cycler MP. Each reaction was carried out in a total volume of 7.5 μL,

Table 1 Characterization of 12 microsatellite loci in the Japanese bush warbler *Cettia diphone*

Locus	Repeat sequence	Primer sequences (5′ to 3′) Upper: Forward, Lower: Reverse	T_a (°C)	Size (bp)	No. alleles	Size range	H_O	H_E	HWE test	Accession number
Cdi1	$T_{13}G(TGCG)_2(TG)_{12}$	HEX – GTACAGGTTTTGGACAGTG CAGTTCCCTCTTCTCCTA	57	157	22	154–194	0.73	0.94	$P < 0.01$	AB089166
Cdi2	$(TTTG)_{11}$	FAM – TATCAGTTGCCAGGAGGG CTGCTCATCACCACCCAA	57	203	14	182–202	0.75	0.87	$P < 0.01$	AB089167
Cdi8	$(AAAAAC)_3AAA(AAAC)_5$	FAM – ACACCCATAGGCAATAGCA GAGAAACCTGCAACAACC	57	298	17	272–295	0.73	0.92	$P < 0.01$	AB089168
Cdi10	$(CAAA)_5 \ldots (CAAA)_2$	TET – AGAGGAACCACCACTGT TGGTTTTGTCAGGGGGT	53	196	9	152–198	0.85	0.74	$P = 0.0529$	AB089169
Cdi25	$(GT)_5(AT)_{23}$	FAM – GCCTGCAAGATGGTGGT CAGTGGATGGCAAGGTG	57	149	19	105–149	0.77	0.93	$P < 0.01$	AB089170
Cdi29	$(CAAA)_2CATA(CAAA)_5$	TET – GGCAAGAAGTGTCAGAAC CATTCATTCCCCTCTCTC	57	161	2	158–162	0.04	0.04	NS	AB089171
Cdi31	$(AG)_{11}$	TET – GAATGAGTGAGCTGGGTC CTAGGCACTCTTGGACAG	57	116	6	103–119	0.54	0.6	NS	AB089172
Cdi32	$(CAAA)_4 \ldots (CAAA)_5$	HEX – TTCCAAGCACTAAGACTG TGCACAAAGCCAACACAGG	53	210	7	195–209	0.27	0.75	$P < 0.01$	AB089173
Cdi35a	$(TTTGG)_4$	TET – GAGCAATGGCAGTTCTACC GCAGGAACAGGAGGAATG	57	244	3	240–247	0.02	0.25	$P < 0.01$	AB089174
Cdi38	$(CT)_{12}$	FAM – ACATCTTCGGCACGGCT GAGCTGGAAGTGGTGGA	57	96	7	87–101	0.76	0.71	NS	AB089175
Cdi39	$(TC)_{12}$	HEX – CTTATCGAGGGCACAGC AGAGGGCAGGGAGTTTG	57	110	8	98–110	0.48	0.56	NS	AB089176
Cdi41	$(GTTT)_5 \ldots (AG)_5AAGG(AG)_4$	FAM – AGATTTCCAGGTCATAGG TTGACTAATCTCTGCTGC	53	183	6	180–185	0.31	0.58	$P < 0.01$	AB089177

T_a: optimized annealing temperature. Size: the size of original clone. H_O: observed heterozygosity. H_E: expected heterozygosity. HWE test: differences between H_O and H_E calculated by GENEPOP. NS: not significant.

containing 20 ng of template DNA, 10 mM Tris-HCl, 50 mM KCl, 2.5 mM $MgCl_2$, 0.25 mM of each nucleotide, 2.5 pmol of each primer, and 0.45 units *Taq* polymerase (Takara). PCR cycles were as follows: 3 min at 94 °C, 30 cycles of 30 s at 94 °C, 30 s at the optimized annealing temperature (Table 1), 45 s at 72 °C, and 72 °C for 10 min Each forward (or reverse) primer was labelled at the 5′-end with either 6-FMT, TET or HEX by the supplier (Applied Biosystems). PCR products were sized using GENESCAN software on ABI 310 according to the manufacture's instructions. In order to check polymorphism and assign the heterozygosity of the loci, 49 presumably unrelated adult individuals captured in Chichibu were analysed. The primer pair for each microsatellite locus amplified PCR products of appropriate length and were polymorphic for the Japanese bush warbler (Table 1). We used GENEPOP for analysis of these data (Raymond & Rousset 1995). The WINDOWS-based computer program GENEPOP was made publicly available and is now a widely used tool by molecular ecologists. Observed heterozygosities (H_O) at five loci (Cdi10, Cdi29, Cdi31, Cdi38 and Cdi39) are not significantly different from expected heterozygosity (H_E) under Hardy–Weinberg equilibrium (HWE) (Table 1). At Cdi1, Cdi2, Cdi8, Cdi25, Cdi32, Cdi35a and Cdi41, there are significant differences between H_O and H_E. Such a deviation from HWE may be a consequence of inbreeding, of a substructuring of the sample or of the presence of null alleles.

We were able to use the 12 primers for analysis of parentage. However, several loci may be unsuitable for this purpose because of their low variability (especially Cdi29 and Cdi35a) or possible existence of null allele (e.g. Cdi41). These polymorphic loci were tested in four other related species on the same conditions. In short-tailed bush warbler *Cettia squameiceps*, Taczanowski's grasshopper warbler *Locustella pleskei* and Ijima's willow warbler *Phylloscopus ijimae*, no loci were amplified. In the great reed warbler *Acrocephalus arundinaceus*, Cdi38 was amplified and polymorphic, but the other 11 microsatellite loci failed to be amplified.

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