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PRIMER NOTE

Nine new tetranucleotide microsatellite markers for the fire-bellied toad (*Bombina bombina*)

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Abstract

We describe nine new polymorphic tetranucleotide microsatellite loci isolated from the fire-bellied toad (*Bombina bombina*). The relative yield of new loci was higher than described in previous studies in amphibians: out of 12 loci initially evaluated, nine were polymorphic and amplifying reliably. Number of alleles ranged from four to 10 and observed heterozygosities from 0.47 to 0.91. Seven loci were polymorphic also in *Bombina variegata* and five in *Bombina orientalis*. Enrichment protocols yielding long flanking regions potentially overcome difficulties (i.e, low yield of reliable loci relative to number of clones screened) which have been reported in microsatellite development in anurans.

Keywords: *Bombina bombina*, cross-species amplification, flanking region, microsatellites, tetranucleotide

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As part of the European Union (EU) LIFE project 'Management of fire-bellied toads (*Bombina bombina*) in the Baltic region' (www.life-bombina.de), we investigate the genetic population structure of this critically endangered species at different geographic scales to improve population-based management. A prescreening using existing primers for dinucleotide microsatellite loci yielded nine polymorphic loci (two from Nürnberger *et al*. 2003; seven from Stuckas & Tiedemann 2006; data not shown), still too few for a reliable population assessment (Takazaki & Nei 1996). We isolated tetranucleotide loci, as these are easier to score. DNA was extracted from toe tips using the QIAGEN tissue kit according to the manufacturer's instructions, except for eluding the samples in only 60-μL elution buffer. Extracted genomic DNA was enriched with two different oligo mixes following the protocol by Glenn & Schable (2005): mix A contained $(AAAC)_6$, $(AAAG)_6$, $(ACAG)_6$, and $(ACGT)_6$, and mix B contained $(AAGT)_8$, $(AGAT)_8$, $(AACT)_8$, $(ACAT)_8$. In brief, the DNA was digested with Rsa*I*, ligated to SuperSNX linkers, hybridized to biotinylated microsatellite oligonucleotides, captured on Dynabeads (Dynal Biotech Inc.), unwanted DNA was washed away, captured DNA was recovered by polymerase chain reaction (PCR) with SuperSNX-f (5′-GTTTAAGGCCTAGCTAGCAGAATC-3′) and cloned using the TOPO TA Cloning System (2.1) (Invitrogen). Colonies were amplified using T3 and T7 primers. PCR products of 500–1000 base pairs (bp) were sequenced using BigDye Terminator version 3.1 chemistry (Applied Biosystems) on an ABI PRISM 3100 sequencer.

Out of 67 clones sequenced, 47 contained microsatellite loci. Primer pairs were designed for 12 loci, out of which nine consistently amplified (Table 1).

Two multiplex PCR amplifications were performed in a 20-μL volume. Final concentrations for optimizing PCRs were 1 × Go*Taq* Flexi Buffer (Promega), 0.22 mM dNTPs, 2.0 mM $MgCl_2$, 0.5 U Go*Taq* DNA Polymerase (Promega) and 2-μL DNA template. Primer concentrations in multiplex reaction A were 0.23 μM of each 11D primer, 0.2 μM 9H, 0.16 μM 8A and 0.135 μM 12F. Multiplex reaction B contained 0.25 μM 5F, 0.165 μM 3A, 0.15 μM 1A, 0.125 μM 7B and 0.1 μM 10F primers. Both reactions were amplified using the same touchdown thermal cycling program encompassing a 10 °C span of annealing temperatures ranging between 60 °C and 50 °C. Following an initial denaturation at 96 °C for 70 s, the temperatures were held for 30 s at 96 °C, 60 °C and 72 °C. Over 30 cycles the annealing temperature was decreased by –0.33 °C per cycle. This was followed by a 15-min extension at 72 °C. Fragment size was determined on an ABI PRISM 3100 automatic

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Table 1 Characterization of nine primer pairs that amplify microsatellites from *Bombina bombina*. Size range refers to the observed distribution of alleles at each locus for 21 toads genotyped for all loci. Number of alleles (N_a), size ranges, observed (H_O) and expected (H_E) heterozygosities, Hardy–Weinberg probability (H–W *P* value) and estimated frequency of null alleles

Locus/primer	Primer sequence 5′–3′	GenBank Accession no.	Repeat sequence	N_a	Size range (bp)	H_O	H_E	H-W *P*-value	Null freq.
Bobom1A.for	ATGTGGCTTCCATTGACCTTTGC	DQ782961	$(GATA)_{12}$	8	342–374	0.86	0.77	0.56	–0.071
Bobom1A.rev	CATGCCAAGAAGGATTGAGTCTGTC								
Bobom3A.for	CTGGAGCCAGCAACTCTGTCTTTAG	DQ782962	$(GATA)_{10}$	6	215–239	0.62	0.8	0.11	0.119
Bobom3A.rev	GAATCGGTTCTTTTATCCTGAAACG								
Bobom5F.for	ATGAATTGGAAGGTAAGAACTTACACC	DQ782963	$(GACA)_{13}GGCA(GACA)_7(GATA)_{14}$	10	203–247	0.91	0.87	0.81	–0.031
Bobom5F.rev	CAAATGATACAAATCAAGTGGAATGG								
Bobom7B.for	GCCCCAAGTCTGTAAATGTATCCAGC	DQ782964	$(GACA)_5(GATA)_7$	5	148–200	0.62	0.73	0.25	0.078
Bobom7B.rev	GGCTGCAATTGGTAACTTGTATGCTAG								
Bobom8A.for	AATTTCTTAGTGCTGCCAACTTGC	DQ782965	$(AGAT)_7AAAGAGAT(GATA)_9$	4	294–306	0.47	0.50	0.52	0.007
Bobom8A.rev	GGGGAAGGGACATTTTAGCTACATAC								
Bobom9H.for	AACAGCCATTATTTAAAACCATTAG	DQ782966	$(GATA)_9TAAA(GATA)_2GAAA(GATA)_6$	8	109–177	0.67	0.77	0.20	0.062
Bobom9H.rev	CAATAAAGCAGTATTTCCCAAAATG								
Bobom10F.for	ATCCAACTTCAAATTCACAGGTCAC	DQ782967	$(GATA)_{12}$	8	199–251	0.86	0.79	0.92	0.048
Bobom10F.rev	ACAAGGGATACCAGGAGAACAAAGC								
Bobom11D.for	CTCTGGTGTTTTGACGTTACTAGGC	DQ782968	$(GATA)_{17}$	6	260–300	0.67	0.66	0.72	–0.009
Bobom11D.rev	CATTTTGCCAAAACACTACTGATAAC								
Bobom12F.for	ATAGGAGGTTTATAATGAAAGGGCAAC	DQ782969	$(GATA)_9$	7	193–229	0.81	0.8	0.35	–0.016
Bobom12F.rev	GATTGGATTTGGGCTATGATATTCTG								

Table 2 Allele ranges of the nine primer pairs for tetranucleotide loci and for three previously isolated dinucleotide loci (Stuckas & Tiedemann 2006) in *Bombina variegata* (*N* = 5), *Bombina orientalis* (*N* = 3), *Alytes obstetricans* (*N* = 1) and *Pelobates cultripes* (*N* = 1). The total number of alleles found is given in parenthesis. A dash indicates no PCR product: indicates loci in *B. variegata* and *B. orientalis* for which Stuckas & Tiedemann (2006) did not obtain PCR products: this locus amplified only in two individuals

Species	Bobom 8A	Bobom 9H	Bobom 11D	Bobom 12F	Bobom 1A	Bobom 3A	Bobom 5F	Bobom 7B	Bobom 10F	Bobom C15	Bobom F22	Bobom D2
Bombina bombina	294–306	109–177	260–300	193–229	342–374	215–239	203–247	148–200	199–251	136–156	148–178	226–232
B. variegata	314–354 (6)	147–223 (6)	247–251 (2)*	206–226 (5)	320–354 (5)	—	130–153 (5)	—	189–229 (6)	—	133–141 (3)	150–225 (5)#
B. orientalis	376–398 (3)	118–182 (4)	—	228–232 (2)	305 (1)	211–239 (2)	—	—	—	197–217 (2)#	142–249 (5)#	185 (1)
Alytes obstetricans	190	185	—	—	306–312	—	—	—	—	—	200–236	221–225
Pelobates cultripes	—	177	—	—	300	227	—	—	—	—	—	225

sequencer using GENEMAPPER version 3.5 and LIZ 500 (Applied Biosystems) as an internal size standard.

Following optimization of multiplex reactions, we genotyped 21 *B. bombina* individuals from a population in northern Germany. We calculated allelic frequencies, observed and expected heterozygosities, analysed deviation from Hardy–Weinberg equilibrium and tested for linkage disequilibrium with GENEPOP (Raymond & Rousset 1995). Frequencies of null alleles were estimated using CERVUS (Marshall *et al*. 1998) (Table 1).

The high numbers of alleles per locus indicate the potential usefulness of these loci to characterize the population genetic structure of *B. bombina*. Observed heterozygosities ranged from 0.47 to 0.91 and did not deviate significantly from expected values. No linkage disequilibrium was detected among any pair of loci, apart from linkage between Bobom1A und Bobom5F; however, this was not found in another population examined ($P = 1.0$; data not shown). Estimated frequencies of null alleles were below 8% for all loci except for Bobom3A. For this locus, almost twice as many individuals as expected were homozygous. However, we were unable to confirm this pattern in another population genotyped (estimated null allele frequency = 0.001; data not shown).

Because these primers might also be useful for population genetic studies in other anurans, we tested them with several individuals of the congeners *Bombina variegata* and *Bombina orientalis*, as well as with one other member of the Discoglossoidea, *Alytes obstetricans*. In addition, we chose one distantly related species, *Pelobates cultripes* (Pelobatidae). We used the same amplification conditions as established for *B. bombina*. Many of these primers produced amplification products of the expected size (Table 2). For the two congeners, several amplicons were sequenced to confirm that they actually contained a microsatellite. Seven of the new loci could be amplified in *B. variegata* and six in *B. orientalis*. According to Stuckas & Tiedemann (2006), only four of eight tested primer pairs for the dinucleotide loci yielded fragments in *B. variegata* and three in *B. orientalis*. As we now had additional DNA samples for these species, we tested three of the dinucleotide loci again. In all but one case, we were able to get amplification products where previous attempts had not been successful (Table 2). We also obtained PCR products for three tetranucleotide and two of the dinucleotide loci in *A. obstetricans* and for three tetranucleotide and one dinucleotide locus in *P. cultripes*. This result is rather promising, as cross-species amplification in anurans is usually considered being limited (Primmer & Merilä 2002).

Stuckas & Tiedemann (2006) attributed their low yield of new loci to the abundance of repetitive sequence motifs in the flanking region ('cryptic simplicity'). However, our 'cryptic simplicity' score was 1.3, not much lower than the 1.5 found by Stuckas & Tiedemann (2006). Instead, our high success may be attributed to a different enrichment protocol (using only one instead of two restriction enzymes). This yielded considerably longer flanking regions (average 519 bp) than those of Stuckas & Tiedemann (2006; average 159 bp) and hence facilitated development of suitable primers.

Apart from the increased statistical power for population analyses, we can evaluate if the two types of microsatellites (dinucleotide vs. tetranucleotide) differ in their variability and therefore contribute differently to genetic structure.

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