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PRIMER NOTE

Eight new microsatellite loci for the critically endangered fire-bellied toad *Bombina bombina* and their cross-species applicability among anurans

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Abstract

We describe eight new microsatellite loci for the critically endangered fire-bellied toad, *Bombina bombina*. Seven of them are polymorphic with two to seven alleles per locus, an expected heterozygosity between 0.41 and 0.8, and an observed heterozygosity between 0.27 and 0.7. The yield of new loci was relatively low, presumably due to mildly repetitive sequence motifs in microsatellite flanking regions. As typical for anurans, cross-species amplification was limited (here, to congeners *Bombina orientalis* and *Bombina variegata*). Combining these new loci with those already available provides a reasonable number of loci for population studies and pedigree analysis in *Bombina*.

Keywords: Anura, *Bombina bombina*, cross-species amplification, Discoglossidae, fire-bellied toad, microsatellites

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The fire-bellied toad (*Bombina bombina*), an anuran species distributed in lowland aquatic habitats of eastern and central Europe, is critically endangered in most of its current distribution range and protected by the so-called 'habitat directive' of the European Union (Council of the European Communities 1992). Standard protocols for microsatellite development often only yield a few new loci, when applied to anurans (e.g. Rowe *et al*. 2000). For the fire-bellied toad, only eight microsatellite loci have been described so far (Nürnberger *et al*. 2003). As the reliability of population genetic assessments critically depends on the number of microsatellite loci studied (Takazaki & Nei 1996), we present here further loci that can be implemented in conservation efforts as well as in further analysis of the stable hybrid zones, which this species forms with the yellow-bellied toad, *Bombina variegata* (e.g. Nürnberger *et al*. 2003).

For microsatellite development, we obtained *B. bombina* tadpoles from laboratory crosses of two Danish populations. We obtained additional samples (phalange tips, tadpole tails or saliva) from nine sites in Germany and Denmark. Cross-species amplification was evaluated in one specimen each of eight other anuran species: Ranidae (*Rana esculenta*, *Platymantis bimaculata*), Microhylidae (*Callupops pullus*, *Oreophryne atrigularis*), Hylidae (*Litoria nigripunctata*), Bufonidae (*Bufo viridis*) and Discoglossidae (*Bombina orientalis*, *B. variegata*).

Genomic DNA of *B. bombina* tadpoles was simultaneously restricted with *Nhe*I, *Hae*II, *Rsa*I and *Alu*I. A microsatellite-enriched genomic DNA library was constructed in Bluescript plasmids and transformed into competent *Escherichia coli* (XL1-Blue MRF', Stratagene), using 5'-biotin-labelled microsatellite probes ($[GA]_{15}$ and $[GT]_{15}$; see Paulus & Tiedemann 2003 for methodical details). Positive clones were sequenced with the BigDye version 1.1 Terminator Cycle Sequencing Kit and analysed on an ABI 3100 automatic sequencer (Applied Biosystems). Primers were constructed from flanking regions of microsatellite loci (Table 1).

Polymerase chain reaction (PCR) was performed in a total volume of 37.5 μL, containing 10 mM Tris-HCl, pH 9.0, 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 0.2 mM of both forward and reverse primers (one of them 5'-fluorescence-labelled) and 0.75 U *Taq* polymerase (QBIOgene). Amplifications were performed in a Biometra

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Table 1 Characteristics of eight microsatellites in *Bombina bombina* and applicability of primers to other *Bombina* species

Primer name and sequence	GenBank Accession no.	Characteristics in *Bombina bombina*								Amplificate sizes in other anurans (— indicates no PCR product)	
		Repeat sequence	T_a	n	No. of alleles	Allele size	H_O	H_E	P	*B. orientalis*	*B. variegata*
BobomF2.for 5′-AGCAGAGATGAGAGGACAGTG	AJ877247	$(GA)_{20}$	60	11	6	264–278	0.55	0.76	0.19	—	480
BobomF2.rev 5′-TCAGGGGTAGCAGATTTTCAG											456
BobomF14.for 5′-ATTCCACTGTCCTTGTTATAATC	AJ877248	$(TC)_{26}C_2(TC)_9$	48	11	4	217–254	0.27	0.41	0.12	—	—
BobomF14.rev 5′-TGAAACCCAGAATACTGAAGC											
BobomD2.for 5′-TTTGAAAAATGTCATGATTAATTCTC	AJ877249	$(TC)_{18}T_2(TC)_{11}$	46	10	7	214–242	0.70	0.68	0.40	186	—
BobomD2.rev 5′-CTTGTAATCCAGCCCTTTATATTTAG											
BobomF22.for 5′-AGGCAAAGGATTCTGAGAATG	AJ877250	$(GA)_{30}$	56	11	6	144–168	0.45	0.59	0.21	—	132
BobomF22.rev 5′-CCTTCAAAGTCGAAAAATATT											
BobomB13.for 5′-ATATTTCTTGCTATGTTGATG	AJ877251	$(GA)_{22}$	46	9	4	121–167	0.67	0.8	0.01	120	126
BobomB13.rev 5′-AATTGTTTAACTTATTTTATA										158	134
BobomC15.for 5′-TTGATATTCCTTTTATTGCCC	AJ877252	$(TC)_{11}T(TC)_6T(TC)_{13}$	53	8	2	142–152	0.38	0.53	0.52	—	—
BobomC15.rev 5′-TTTCCCTAATGCTAATTATGG											
BobomO9.for 5′-AAGGTAATAACACACACTCCC	AJ877253	$(AC)_8TCATACA(AC)_{24}$	58	9	2	204–210	0.44	0.39	1.00	—	—
BobomO9.rev 5′-GGTCATTTGAAAAGTAGATCC											
BobomB14.for 5′-ACTAACCTGCCACATAACTTG	AJ877254	$(TC)_4T(TC)_7T(TC)_9GC(TC)_7$	48	11	1	161	0	0	1.00	167	184
BobomB14.rev 5′-CTGGGTTTTTTAATTGGAAGG										171	186

T_a (°C), PCR annealing temperature; n, no. of *Bombina bombina* specimens analysed; H_E, expected heterozygosity; H_O, observed heterozygosity; P, probability for Hardy–Weinberg equilibrium.

 Molecular Ecology Notes, 10.1111/j.1471-8286.2005.01171.x

TGradient thermocycler according to the following reaction profile: initial denaturation at 94 °C for 5 min, 3 cycles: 94 °C for 1 min, an elevated locus-specific annealing temperature (T_a + 3 °C, Table 1) for 1 min, 72 °C for 1 min; 38 cycles: 94 °C for 1 min, T_a °C (Table 1) for 1 min, 72 °C for 1 min; and a final extension at 72 °C for 40 min. Fragment size was determined on an ABI 3100 automatic sequencer using GENEMAPPER version 3.5 and an internal size standard (Map Marker, Eurogentec; or LIZ 500; Applied Biosystems).

We identified 103 clones containing microsatellite loci. In 40 cases, sequence information of flanking regions was sufficient for primer design, performed with the OLIGO version 3.4 software (Rychlik & Rhoads 1989). Of these 40 primer pairs, eight reliably amplified the expected microsatellite locus, as confirmed by sequence analysis (Table 1). Seven loci were polymorphic in *B. bombina* and showed two to seven different alleles. Observed heterozygosity (ranging from 0.27 to 0.7) was not significantly different from expected heterozygosity (0.39–0.8), except for locus BobomB13 (calculated and tested using ARLEQUIN; Schneider *et al*. 2000; Table 1). Using the same software and a Bonferroni correction to adjust for multiple pairwise comparisons, no linkage disequilibrium was detected among any pair of loci, apart from linkage between BobomF22 and BobomD2.

As previously found in frogs (Primmer & Merilä 2002), cross-species amplification was limited: our new loci never worked in other anuran genera, but some amplified in the congeners *B. orientalis* and *B. variegata*. Interestingly, locus BobomB14 was polymorphic in these species, while monomorphic in *B. bombina* (Table 1).

Only 20% (8/40) of our newly designed primer pairs (20%) reliably amplified a single microsatellite locus. This low yield is in line with previous studies (Nürnberger *et al*. 2003). It is potentially caused by repetitive sequence motifs in the flanking region of microsatellites. To test for repetitive sequence motifs in our data, we evaluated the complete flanking regions of the 40 cloned microsatellite loci (ranging from 15 bp to 463 bp; 9093 bp in total) with the N-simple algorithm (Alba *et al*. 2002; settings: window of 32 bp, 100 simulations). In short, this algorithm moves a window of a defined size (here, 32 bp) over a sequence region, bp-by-bp. For each such window, (i) the bp composition is scored, and (ii) the occurrence of the central motifs of one to four bp length is evaluated, relative to the expected occurrence of such repeats in randomly generated sequences with the same bp composition (Alba *et al*. 2002). Indeed, this so-called 'cryptic simplicity score' was 1.5 in our data, statistically significantly larger than 1, the random expectation. Due to repetitive motifs in the flanking regions and the low rate of cross-species amplification, numbers of available microsatellite loci are often constrained in anurans. In our case, taking advantage of two microsatellite screens (Nürnberger *et al*. 2003; this study) provides a reasonable number of loci for population studies as well as pedigree analysis in the genus *Bombina*.

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