

PERMANENT GENETIC RESOURCES

Eight new tetranucleotide microsatellite loci for the agile frog (*Rana dalmatina*)

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Abstract

We describe eight new polymorphic tetranucleotide microsatellite loci isolated from the agile frog (*Rana dalmatina*). In 25 individuals from the Nature Reserve Lüneburger Heide (Lower Saxony, Germany), the number of alleles per locus ranged from four to nine and average observed heterozygosities from 69.1% to 80.7%. No evidence for linkage disequilibrium was found and none of the loci showed significant deviation from Hardy–Weinberg expectations. These microsatellite DNA markers are suitable tools for addressing population genetics issues in this endangered species.

Keywords: anura, microsatellites, motif, *Rana dalmatina*, tetranucleotide

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The agile frog (*Rana dalmatina*) is widespread in Europe, ranging from eastern Spain to Romania, and from Denmark to southern Greece. In Germany, the agile frog has a rather disjunct distribution: its largest occurrences are in central and southern Germany. In northern Germany, its distribution is very patchy: it is found only in two coastal areas on the Baltic Sea (Rügen and Darß), north of the Harz Mountains, and in the Lüneburger Heide, a heathland area north of Hanover (Günther 1996). *Rana dalmatina* is listed as an endangered species within the Fauna-Flora-Habitat (FFH) directive appendix IV. During the course of a larger project launched to establish standardized DNA markers as a set of species-specific microsatellite loci for threatened German amphibian species, we describe here the isolation of polymorphic tetranucleotide microsatellite loci for *R. dalmatina*. These new microsatellite loci will be helpful to address questions at the interface of FFH-relevant monitoring and conservation issues.

From our own experience (Steinfartz *et al.* 2004; Hauswaldt *et al.* 2007), we have found microsatellite loci with tetranucleotide motifs easier to score and therefore aimed at isolating tetranucleotide loci. For the enrichment, genomic DNA was used from an individual *R. dalmatina*

collected near Cologne, Germany. DNA was enriched with two oligo mixtures containing tetranucleotide probes following the protocol by Glenn & Schable (2005). Mix A contained (AAGT)₈ (AGAT)₈ (AACT)₈ (ACAT)₈ and (AAAT)₈ as probes, mix B contained (AAAC)₆ (AAAG)₆ (AATC)₆ (ACAG)₆ (ACTC)₆ and (ACTG)₆. In brief, genomic DNA was digested with *RsaI*, ligated to SuperSNX linkers, hybridized to biotinylated microsatellite oligonucleotides and captured with Dynabeads (DynaL Biotech Inc.). Unspecifically bound DNA was washed away according to the protocol and specifically bound DNA was recovered by polymerase chain reaction (PCR) with the SuperSNX-f (5'-GTTAAGGCCTAGCTAGCAGAATC-3') primer. PCR products were enriched for the specific microsatellite motifs (see above) an additional time using the same procedure and conditions as described before. The resulting PCR products were cloned using the TOPO TA Cloning System (2.1) (Invitrogen). Inserts from colonies were amplified using M13 primers. Altogether, 65 colony-PCR products of 500–1000 bp were sequenced with T3 and T7 primers using BigDye version 3.1 chemistry (Applied Biosystems) on an ABI 3730 automated sequencer. Of these, 57 were from the enrichment with mix A and eight from the enrichment with mix B.

Forty clones contained microsatellite motifs with at least six repeats units. Fifteen of these were not considered for further analysis as they contained mixtures of di- and

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Table 1 Characterization of eight new microsatellite loci for *Rana dalmatina*

Locus	Primer sequence 5'→3'	GenBank Accession no.	Repeat sequence	Size		P value			
				N_A	range (bp)	H_O	H_E	HWE	PIC
Radal-B5-F	FAM-CAGTAGTTCGTGTACACGGGAAC	EU364510	(TATC) ₁₁	5	380–404	0.60	0.72	0.05	0.650
Radal-B5-R	ACCCTTGAGCATAATGGAATAGC								
Radal-C8-F	FAM-CTTTCTACCCATCTATCCCATATC	EU364511	(TATC) ₁₂	4	182–194	0.80	0.76	0.91	0.758
Radal-C8-R	TGGAAACATTCATGGAAATACG								
Radal-E8-F	AGCTAGATGGGTTTCAAAATGC	EU364512	(TATC) ₁₇ G(TATC) ₈	8	366–414	0.76	0.74	0.35	0.682
Radal-E8-R	FAM-TGAGAGATCAGACAACTAAAGATAGG								
Radal-F3-F	FAM-TTATATTCGGGTTCGGCTTATACTC	EU364513	(TATC) ₆ (TGTC) ₃ GTTC(TATC) ₁₆	9	213–257	0.76	0.81	0.24	0.762
Radal-F3-R	AAAATGTATCCGCACCAAATAC								
Radal-F5-F	FAM-GTAAAGCTCTCATGAAACCTAAAAG	EU364514	(TAGA) ₉ (TAGG) ₂ TAGA(TAGG) ₂ (TAGA) ₆	4	157–173	0.60	0.70	0.85	0.703
Radal-F5-R	TGCATATGCTGACTGTATTTATTTG								
Radal-G11-F	HEX-GAAATAAACTTCCTGGTGGTTGG	EU364515	(GATA) ₁₁	5	351–367	0.60	0.76	0.06	0.696
Radal-G11-R	TCCAGTGCCTAATCTCTCTCTC								
Radal-G12-F	HEX-TTTGGAATTCATGTCCAACAGG	EU364516	(TAGA) ₁₀ TAAA(TAGA) ₅	8	235–263	0.84	0.81	0.62	0.759
Radal-G12-R	CAGAGACATTTTGTCTGGTTTG								
Radal-H1-F	FAM-CCGGAACAAATATTTTCAAGTGTG	EU364517	(CTAT) ₁₆	5	172–192	0.76	0.69	0.46	0.623
Radal-H1-R	GGTGGGCACTAGAAAAGAAATAAAG								

Number of alleles (N_A), size range, observed (H_O), expected heterozygosity (H_E), Hardy–Weinberg probability (P value HWE), and polymorphic information content (PIC) are presented for each locus and are based on 25 individual frogs genotyped at all loci.

tetranucleotide motifs, and in four other clones, the flanking region of the microsatellite locus was too short for successful primer design. Therefore, primers were designed for 21 loci using the PRIMER 3 software (version 0.4.0; <http://frodo.wi.mit.edu/primer3/input.htm>). Of these, seven loci were not variable. Primer pairs for seven other loci did not render amplicons of the appropriate size or generated more than two bands per individual. Primers for eight polymorphic loci were identified, all of which were derived from the enrichment with oligo mix A (Table 1).

Three multiplex and one single locus PCR were performed using the Multiplex PCR Kit (QIAGEN) in 10- μ L reactions. Reactions contained 3.0 μ L Master Mix, 0.4 μ L of each primer (10 μ M) and 1 μ L DNA (10–20 ng/ μ L). Radal-H1 and G11, as well as B5 and C8 were amplified in duplex reactions. Radal-E8, F5 and G12 were amplified in a triplex reaction, and Radal-F3 by itself. All reactions were performed using the same thermal cycling programme: following an initial denaturation at 95 °C for 15 min, 45 cycles were run with 95 °C for 30 s, 60 °C for 90 s and 72 °C for 60 s followed by a 10-min extension at 72 °C. Fragment sizes were determined on an ABI 3730 automated sequencer using Genescan LIZ 500 (Applied Biosystems) as an internal size standard and genotyped using GENEMARKER version 1.51.

We genotyped 25 *R. dalmatina* individuals from the Lüneburger Heide in Germany and calculated allele frequencies, observed and expected heterozygosities, and polymorphic information content (PIC) values using CERVUS

version 3.0 (Kalinowski *et al.* 2007). We further analysed the loci for deviation from Hardy–Weinberg Equilibrium and tested for linkage disequilibrium with GENEPOP Web version 3.4 (Raymond & Rousset 1995) using the default number of permutations.

Observed heterozygosities ranged from 0.60 to 0.84 and did not deviate significantly from expected values. No linkage disequilibrium was detected among any pair of loci and none of the loci deviated from Hardy–Weinberg equilibrium. Estimated frequencies of null alleles were below 11% for all loci. Therefore, this set of loci will be applicable for paternity analysis as well as suitable addressing population genetic issues.

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