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PRIMER NOTE

Isolation of new microsatellite loci from the Green Lizard (*Lacerta viridis viridis*)

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Abstract

Twelve new microsatellite loci were isolated from the Green Lizard (*Lacerta viridis viridis*). Primers for 28 loci were designed and 18 of these loci amplified well for 10 individuals of four populations. Twelve of these loci were further characterized for a population in Hungary. The results document the suitability of these identified loci for the characterization of the genetic diversity of the endangered species *L. viridis viridis*.

Keywords: *Lacerta viridis*, Lacertidae, microsatellites, polymorphism

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Lacerta viridis viridis is a lizard, which occurs mainly in eastern Europe with isolated populations in northeast Germany. It is a species, which is highly endangered in Europe and many efforts have been undertaken to protect this species (Elbing & Nettmann 2001). While current programs try to artificially recolonize protected areas with *L. v. viridis*, information on the genetic diversity of natural populations becomes a necessity. Therefore, to eventually test the genetic diversity throughout the whole species range of *L. v. viridis*, we identified 12 microsatellite loci. We used these loci to test the genetic diversity of a selected population of *L. v. viridis* in Hungary, which is central to its species range.

For the enrichment of the genomic library we used a modified FIASCO protocol described in Zane *et al*. (2002). Genomic DNA was isolated using a DNeasy Tissue Kit (Qiagen). After isolation, 0.1 μg genomic DNA was used for a digestion-ligation reaction with *Mse*I and *Mse*I AFLP adaptors (*Mse*I-Ad1 5′-TACTCAGGACTCAT-3′ and *Mse*I-Ad2 5′-GACGATGAGTCCTGAG-3′). The digestion-ligation reaction was incubated for three hours at 37 °C, followed by an amplification using adaptor-specific primers to maximize total DNA amount, according to Zane *et al*. (2002). To view the size range, products were run on a 1.5% agarose gel, which showed a smear ranging from 300 up to 1000 bp. These fragments were hybridized with eight different 3′ biotinylated probes (AG_{10}, AT_{10}, AC_{10}, GA_{22}, CA_{22}, AGG_{10}, AAT_{15}, $AGAT_{11}$). The probes were designed with additional four nucleotides (GCAC) on the 3′ end of the repeat. Adapter-ligated DNA fragments (0.6 μg) were hybridized with a 65 pmolar biotinylated probe within a total volume of 100 μL containing 4.2 × SSC and 0.07% SDS. The reaction mix was denatured at 95 °C for 3 min and then allowed to cool down to room temperature and placed on a rocking platform for 30 min at room temperature. Thereafter, washed streptavidin magnetic particles (1 mg, Roche) and 300 μL of TEN100 (10 mm Tris-HCl, 1 mm EDTA, 100 mm NaCl, ph 7.5) were added to the hybridization mixture and gently mixed for about 30 min at room temperature. Non-specific DNA fragments were removed by washing the mixture three times with TEN1000 (10 mm TRIS-HCl, 1 mm EDTA, 1 m NaCl, ph 7.5) followed by three steps of washing in 0.2 × SSC/0.1% SDS buffer at room temperature. After washing, the separated beads were re-suspended in 10 × TE buffer and denatured at 95 °C for 5 min. The supernatant containing the DNA was removed and precipitated with one volume of Isopropanol and sodium acetate (0.15 m final concentration).

Microsatellite containing DNA was amplified with adaptorspecific primers within a total volume of 20 μL containing 1 × buffer (10 mm Tris-HCl, 50 mm KCl, 1.5 mm $MgCl_2$, pH 8.3; SIGMA), 0.2 mm dNTPs, 6.5 pmol of each primer and 0.5 U *Taq* DNA Polymerase (SIGMA). The polymerase chain reaction (PCR) was performed on an Eppendorf Mastercycler for 30 cycles at 95 °C for 30 s; 53 °C for 30 s; 72 °C for 30 s and one final step at 72 °C for 10 min. The ligation of the enriched fragments was carried out with a TOPO-TA cloning kit (Invitrogen) and plasmids

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Table 1 Microsatellite loci for *Lacerta viridis viridis* based on 27 individuals from Hungary (Gödöllö)
T_a = annealing temperature; H_O = observed heterozygosity; H_E = expected heterozygosity

Locus	GenBank accession no.	Primer Sequences (5′–3′)	Approx. Motiv	size (bp)	No. of T_a (°C)	Alleles	H_O	H_E
Lvir1	AJ783621	F: FAM-TCCGCCTTCCATGGCCAATCC R: CGCTTGAGAGAAAACGGAAACTG	$(TC)_7(TG)_{12}$	349–397	55	7	0.56	0.64
Lvir2	AJ783622	F: NED-ACACATCGCACCAACACGCAG R: TCTGCTTCAGCATCCGTGGAG	$(CA)_8$ … $(CA)_3$	392–406	55	4	0.56	0.42
Lvir4	AJ783623	F: FAM-GCAAAGCATGGAGCTAGCCAC R: TCACACTGCTCTGAATGCCAG	$(CA)_{17}$	357–379	55	11	0.67	0.77
Lvir6	AJ783624	F: HEX-CTAGGGTTTAGAGGGGCTTCC R: TCCTGTCGCTCCACCGAATGC	$(TC)_8$ … $(CTAT)_{13}$ … $(CT)_7$	425–465	55	11	0.70	0.87
Lvir7	AJ783625	F: HEX-TCGACAGCTTGCAGGCTTGAC R: GAAGGGCTCTTCCAGACACTG	$(CTTT)_{15}$	352–440	55	15	0.89	0.87
Lvir9	AJ783626	F: NED-TGAGATCCTTATTGGCAGACTCC R: ACTTCCAAGCTGCACCGGCTC	$(TATC)_{10}$ … $(TATC)_{10}$	340–378	55	11	0.85	0.88
Lvir10	AJ783627	F: HEX-GTGGAGCCTAAGCGAAACCGCAC R: TGCTCGCAAACGTCTTGGGCTGG	$(TCTCC)_5$	345–410	55	6	0.48	0.63
Lvir11	AJ783628	F: NED-GCATGACCTCTGCCTACGCAG R: TCCTGAGGGCCTGAAAGC	$(CTTT)_7(CCTT)_3$	280–288	55	3	0.33	0.38
Lvir14	AJ783629	F: HEX-CTGGGTGACCTTGGGCTAGTC R: ATTGCTGAGGTGCAGAACTGC	$(TTC)_6(CTT)_{15}$	367–445	55	20	0.96	0.93
Lvir16	AJ783630	F: FAM-GAGACCGGAGTTGGCAGTTGGC R: GGATACTAATGGCTTTTCTCC	$(TC)_{25}$ … $(TG)_{12}$	258–298	55	12	0.74	0.85
Lvir17	AJ783631	F: NED-AGCTCTGGATCGAGACAACCTGG R: TCTCTGAAGGAGACCGGCTCC	$(GATA)_{13}$ … $(GA)_{26}(TG)_4$	334–398	55	20	0.70	0.90
Lvir18	AJ783632	F: FAM-GGTGGGTAGCCGTGTTGGTCTGC R: TGGCACAGAATACCAGCACCTCG	$(CT)_{10}$	284–302	58	5	0.59	0.60

were transferred into chemically competent TOP10′F cells (Invitrogen). Seventy-nine positive clones were sequenced using the ABI PRISM BigDye v. 3.1 Cycle Sequencing Kit and M13 primers and analysed with an ABI Prism 3100 automated sequencer, following the manufactures protocol. Repetitive sequences were found within 82% of these positive clones. Primers were designed for the flanking regions of 28 repetitive sequences using FASTPCR (Kalendar 2003). Microsatellite variability of these 28 loci was tested in about 10 individuals from four populations throughout the geographical range of *L. v. viridis*. Of the tested microsatellite loci, 64% displayed a variation and of these, 12 loci were chosen and fluorescent primers were designed accordingly. Forward primers were 5′ labelled with 6-FAM, HEX (MWG-Biotech) and NED (ABI). To inhibit the occasional addition of an adenosine base to the end of the amplified fragment we added a seven base 'pigtail' (GTTGCTT) to the 5′ end of the reverse primers according to Brownstein *et al*. (1996). The PCR was performed in a total volume of 25 μL with 1 × buffer (10 mm Tris-HCl, 50 mm KCl, 1.5 mm $MgCl_2$, pH 8.3; SIGMA), 1.5 mm $MgCl_2$, 0.2 mm dNTPs, 10 pmol each primer and 0.5 U *Taq* polymerase (SIGMA). Microsatellite amplification was performed on an Eppendorf Mastercycler using the following profile: initial denaturation at 95 °C for 1 min; 30 cycles at 94 °C for 30 s; at the locus specific annealing temperature for 30 s; and at 72 °C for 30 s.

Genotyping of fluorescent fragments was performed with an ABI Prism 3100 automated sequencer using GeneScan-500 ROX size standard (ABI). Allele size was called with the ABI PRISM GENESCAN Analysis Software v 3.7.1 and brought to a round figure. All loci were polymorphic in an analysis of 27 individuals from a Hungarian population. Analysis of genetic diversity was carried out using GENALEX (Peakall & Smouse 2001) and GENEPOP (Raymond & Rousset 1995). The loci had three to 20 alleles and expected heterozygosities of 0.42–0.93 (Table 1). Three of the loci displayed significant heterozygote deficiencies (χ^2-test; $P < 0.05$). This may be due to null alleles or an insufficient sampling size. Furthermore, the loci Lvir16 and Lvir4, Lvir10 and Lvir6, Lvir9 and Lvir6 show linkage disequilibria with low significance values. All of the microsatellite loci presented here will be used to analyse the pattern of genetic variability throughout the species range *L. v. viridis*.

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