Furthermore, after Bonferroni correction, there was no indication of linkage disequilibrium among loci.

The multiplex kits were also tested on two individuals from nine cyprinid species. Amplifications were successful in 87.3% cases (Table 2), highlighting the potential usefulness of these markers for a wide range of population studies in European cyprinids including phylogeographical approaches and conservation issues.

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Isolation and characterization of polymorphic microsatellite loci for the Skyros wall lizard *Podarcis gaigeae* (Squamata: Lacertidae)

M. WELLENREUTHER, A. RUNEMARK, E. I. SVENSSON and B. HANSSON
Department of Animal Ecology, Ecology Building, Lund University, SE-22362 Lund, Sweden

Abstract

Fifteen polymorphic markers were developed from a microsatellite-enriched library for the lizard *Podarcis gaigeae*. The loci were checked for variability in 68 individuals from a population on the island of Skyros, Greece. The number of alleles ranged from 3 to 23 per locus and expected heterozygosity from 0.29 and 0.94. Most markers were also polymorphic in three closely related *Podarcis* species, namely *P. erhardi*, *P. taurica* and *P. milensis*. The markers will be used to examine gene flow and differentiation of island and mainland populations of *P. gaigeae*.

Keywords: gene flow, microsatellite markers, molecular markers, *Podarcis gaigeae*, population differentiation, Squamata

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Lizards of the lacertid genus *Podarcis* are widespread throughout Europe and North Africa. *Podarcis gaigeae* is endemic to the archipelago of Skyros, Greece (Poulakakis *et al*. 2005), and exhibits substantial morphological differentiation between islands (A. Runemark, unpublished). This pattern of morphological differentiation over small spatial scales makes this species an interesting model for understanding processes that promote divergence. Previously, Runemark *et al*. (2008) have identified 11 polymorphic microsatellite marker for *P. gaigeae*, and these markers were subsequently used to identify the population structure of mainland and island populations of this species. Preliminary results showed that although the markers were able to distinguish between different island populations, they did not provide the necessary power to distinguish between the mainland populations (A. Runemark, unpublished). Therefore, we set out to develop 15 novel polymorphic microsatellite markers for *P. gaigeae* in an attempt to resolve the population structure of the mainland populations, and to estimate the magnitude and direction of gene flow between mainland and island populations of this species in Greece.

Tail samples of *P. gaigeae* were collected from a population of the mainland of Skyros and preserved in 70% ethanol. Genomic DNA was extracted with an ammonium acetate extraction protocol (Richardson *et al*. 2001). A microsatellite-enriched library was constructed using the protocol provided by Zane *et al*. (2002) with slight modifications following Nordström & Hedren (2007). Briefly, 500 ng genomic DNA of four individuals was digested with 10 U of Bsp1431 (Fermentas) together with 1× reaction buffer (Fermentas) and 1 μg BSA in a total volume of 20 μL. The reaction mix was incubated for 1 h at 37 °C and, to deactivate the enzyme, for 20 min at 80 °C. The ligation reaction contained 5 μM adaptor (5′-GTGGTAGACTGCGTACC-3′ and 5′-GATCGGTACG CAGTCTAC-3′), 1× ligation buffer (USB) and 0.5 U of T4 DNA ligase (USB). Five-microlitre ligation reaction mix was added to the digestion reaction and incubated for 3 h at 37 °C. The digestion–ligation mix was diluted 1:9, and 10 μL was amplified using the following conditions: 0.5 μm specific primer (5′-GACTGCGTACCGATC-3′), 2 mM $MgCl_2$, 4 μm dNTPs, 2× polymerase buffer (Applied Biosystems), 0.5 U AmpliTaq DNA polymerase (Applied Biosystems). Polymerase chain reaction (PCR) conditions were as follows: 94 °C 30 s, 56 °C 1 min, 72 °C 1 min, for 25 and 30 cycles. The samples with the most evenly amplified smear from the 25- and 30-cycle PCR's were pooled and used for the library construction. Pooled PCR products were hybridized with 100 pM of a mixture of biotinylated oligonucleotides probes: $(AG)_{21}$, $(ATT)_6$, $(AT)_7$, $(AAG)_8$ and $(AAAT)_7$. Streptavidin-coated beads (Roche) were then added and incubated at room temperature to capture DNA fragments with microsatellite sequences complementary to the oligonucleotide probes. Beads and attached probes were separated magnetically from the supernatant using a Magnetic Particle Separator (Roche). Following three soft and three hard stringency washes, the bound DNA was recovered by incubating in Tris-Low-EDTA at 95 °C for 5 min.

The TOPO TA Cloning Kit (Invitrogen) was used to clone fragments according to the manufacturer's instructions. Colonies with inserts were identified by white colour and suspended in 150 μL distilled water and heated at 95 °C for 3 min. When analysing the white colonies by PCR, the following conditions were used: 0.4 μM forward primer (5′-TGTAAAACGACGGCCAGT-3′), 0.4 μM reverse primer (5′-CAGGAAACAGCTATGACC-3′), 1.5 mM $MgCl_2$, 125 μM dNTPs, 1× polymerase buffer (Applied Biosystems), 0.5 U AmpliTaq DNA polymerase (Applied Biosystems), 2 μL template in a final volume of 25 μL. The PCR conditions were as follows: 94 °C for 30 s, 50 °C for 1 min, 72 °C for 1 min 30 s, for 35 cycles. The PCR products were subsequently precipitated and diluted in 10 μL ddH_2O. Two microlitres of this solution was used as template DNA in a sequencing reaction using the BigDye Terminator version 1.1 Cycle Sequencing Kit (Applied Biosystems) together with the forward primer. The sequencing PCR programme was set as follows: 96 °C for 10 s, 50 °C for 5 s, 60 °C for 4 min, for 25 cycles. Precipitated products were sequenced on an ABI PRISM 3100 genetic analyser (Applied Biosystems).

Ninety-six inserts were sequenced and 30 contained microsatellite motifs. Twenty-four primer pairs were designed using the program Primer 3 (Rozen & Skaletsky 2000) and 15 of them proved to be polymorphic in seven test individuals. The remaining loci were either monomorphic, did not amplify or had nonspecific products (data not shown). The variability of the polymorphic primers was evaluated in 68 individuals from the Skyros mainland population Nyfi (38°48′31, 24°34′10) using a touchdown M13(–21) PCR protocol (Schuelke 2000). The PCR mix contained 0.01 μM M13-tailed F-primer, 0.3 μM dye-labelled (6-FAM, HEX, or NED) M13-primer (5′-CACGACGTTGTAAAACGAC-3′), and 0.4 μM R-primer, 15 nM $MgCl_2$, 1.25 nM dNTP, 0.5 U AmpliTaq polymerase and 2 μL DNA in a 10-μL reaction. PCRs were done in a GeneAmp 9700 thermocycler (Applied Biosystems): 94 °C for 2 min; 5 cycles at 94 °C, 60 °C ($\Delta = -0.5$ °C/cycle), 72 °C for 30 s each; then 24 cycles of 94 °C, 58 °C, 72 °C for 30 s each; 15 cycles of 94 °C, 53 °C, 72 °C for 30 s each; followed by 72 °C for 10 min. PCR products were separated in an ABI PRISM 3730 capillary sequencer (Applied Biosystems).

Diversity measures and deviations from Hardy–Weinberg expectations were computed using the software FSTAT (Goudet 1995), and tests for null alleles were conducted using Micro-Checker (Van Oosterhout *et al*. 2004). The number of alleles per locus ranged from 3 to 23 with a mean of 11.9

Correspondence: M. Wellenreuther. Fax: +46 (0) 46 2229014; E-mail: Maren.wellenreuther@zooekol.lu.se

Table 1 Information for the 15 novel microsatellite markers for *Podarcis gaigeae*, including GenBank Accession numbers, microsatellite repeat sequence, primer sequences, number of alleles (A), observed and expected heterozygosities (H_O and H_E), Hardy–Weinberg probabilities (HWP), product size ranges and number of individuals that produced interpretable products (A). HWP values that significantly deviate from the Hardy-Weinberg equilibrium after Bonferroni correction are indicated (*)

Locus	GenBank Accession no.	Repeat motif	Primer sequences (5′–3′)†	A	H_O	H_E	HWP	Size range (bp)	*N*
P011	FJ483918	$(GT)_{10}$	F: AGTGCTTTGCTCCCGTTACC R: GCGGATCACGGTGACACTAA	18	0.823	0.837	0.473	192–248	65
P013	FJ483919	$(CTT)_{14}$	F: GAAGGGAGGAAGGGAAAGGA R: TCTTGATGTGCCCATTTTGC	12	0.841	0.862	0.247	207–263	62
P018	FJ483920	$(CTT)_{13}$	F: GGACTCCAACTGGAGAACAGC R: ACCTTGGGCCAGTCACACTT	13	0.882	0.854	0.684	214–253	58
P020	FJ483921	$(GT)_3T(GT)_{13}$	F: GAGGGAGGGGAGAGGAAATG R: ATTTGGCTCAGCCCCAGTAA	13	0.754	0.838	0.066	247–277	64
P022	FJ483922	$(AC)_{20}$	F: CAGGACAGGAAAGGGCAGAG R: TCCACTATGCAAGGAGCCACT	11	0.815	0.867	0.115	341–363	68
P024	FJ483923	$(CTT)_{13}$	F: AGGTGGGGGTGAGCATAACA R: GCTGGCTCAGGGTGAGAACT	23	0.781	0.937	< 0.001*	184–263	61
P042	FJ483924	$(TC)_7TTG(C)_{12}$	F: CTGGCCGAAAATGGACAACT R: ACATTTCCACACGCAAGCAC	8	0.519	0.656	0.012	247–262	68
P043	FJ483925	$(CTT)_{16}$	F: CACATGACCCGGAAGCTGTA R: CCCTTCTGCCACACAAGACA	19	0.858	0.929	0.043	279–333	65
P046	FJ483926	$(TC)_{20}(GT)_9$	F: CCTCCGCAATGTTTGAGAAA R: GAATACATCTGGGGCGCTTT	18	0.910	0.886	0.764	333–426	65
P047	FJ483927	$(CA)_7$	F: TGCAGTCAGGGGATGAACAG R: GTCCTTCGAGGAATGCCAAG	3	0.247	0.294	0.129	130–136	65
P051	FJ483928	$(CTT)_5CCT(CTT)_6$	F: AGCCGCTTTGAGACTCCTTC R: CAATAGCCCTCTCCCCTGTG	3	0.661	0.604	0.655	170–176	65
P054	FJ483929	$(CA)_{16}$	F: CGGTACAATGATGCTGCAAA R: TGGAAGTTTCCATTGATGTTGC	15	0.832	0.903	0.124	101–129	67
P055	FJ483930	$(CAA)_6$	F: TGGCATCTGAGGCAGAAAAA R: CCTCAGGCAGCAGATTTTGA	7	0.797	0.627	0.997	144–164	68
P056	FJ483931	$(CA)_{20}$	F: TCCCAAAGCAAGAGGTAGCA R: TGTTTTTAATGTTTATATGCCCAGGA	11	0.827	0.883	0.098	131–157	68
P076	FJ483932	$(TG)_6$	F: GGCAGATTTGCTGGTAAATGG R: TCCTCAACCACCCAGGTCTT	12	0.827	0.863	0.339	267–279	64

†M-13 (CACGACGTTGTAAAACGAC) tail attached to the 5′ end of the forward primers.

alleles, and the fragment size varied from 101 to 426 bp (Table 1). Observed and expected heterozygosity levels ranged from 0.25 to 0.91 and from 0.29 to 0.94, respectively (Table 1). Results from Micro-Checker suggested the presence of null alleles in PO24, PO42 and PO78 (Van Oosterhout estimate), and PO24 also showed deviations from Hardy–Weinberg equilibrium after Bonferroni correction. The null allele frequency of these three loci was estimated to range between 6.6% and 8.4%. Tests of linkage equilibrium between all pairs of loci were performed in Arlequin (Schneider *et al*. 2000) and showed that one pair of loci deviated significantly after Bonferroni correction (P047 and P051; adjusted nominal level $P = 0.0001$). Cross-amplification tests showed that the majority of markers were also polymorphic in *P. erhardi*, *P. taurica* and *P. milensis* (Table 2). Thus, most of these microsatellites appear to be useful and reliable for investigations of gene flow and population structure of *P. gaigaea*, and they form a valuable genetic resource for the related species *P. erhardi*, *P. taurica* and *P. milensis*.

Table 2 Cross-amplification success for *Podarcis erhardi* ($n = 12$), *P. milensis* ($n = 11$) and *P. taurica* ($n = 4$). The number of alleles for each locus is given, and the number in brackets denotes number of successful amplifications. NA denotes primers that did not amplify in a species

Locus	*P. erhardi*	*P. milensis*	*P. taurica*
P011	7(8)	4(10)	2(3)
P013	4(7)	7(8)	4(2)
P018	5(9)	3(3)	NA
P020	1(11)	6(10)	1(3)
P022	10(12)	8(11)	2(2)
P024	8(11)	1(1)	NA
P042	6(8)	3(11)	2(4)
P043	NA	NA	6(4)
P046	2(7)	6(10)	1(1)
P047	2(12)	2(11)	3(4)
P051	1(2)	1(1)	NA
P054	7(12)	10(11)	2(4)
P055	1(3)	3(11)	3(4)
P056	2(2)	2(8)	5(4)
P076	2(12)	7(11)	4(4)

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Microsatellite markers for the ectomycorrhizal basidiomycete *Lactarius mammosus*

N. HÖGBERG,* A. GUIDOT,*† M. JONSSON* and A. DAHLBERG*‡

**Department of Forest Mycology and Pathology, Swedish University of Agricultural Sciences, PO Box 7026, S-750 07 Uppsala, Sweden, †Laboratoire d'Ecologie Microbienne (UMR CNRS 5557), Université Claude Bernard Lyon 1, Bât. A. Lwoff, 43 Bd. du 11 Novembre 1918, F-69622 Villeurbanne Cedex, France*

Abstract

We report the characterization of polymorphic microsatellite markers in the ectomycorrhizal fungus *Lactarius mammosus* Fr. Two enrichment protocols were used to isolate microsatellite loci and polymorphism was explored within 31 sporocarps originating from a forest site in northern Sweden. We found nine variable microsatellite loci with the number of alleles per locus varying between 2 and 5, and expected heterozygosities ranging from 0 to 0.84. These loci are available for the analysis of genetic structure and gene flow in *L. mammosus* populations.

Keywords: ectomycorrhizal fungus, *Lactarius*, microsatellite

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Correspondence: N. Högberg. Fax: +46 (0) 18 67 35 99; E-mail: nils.hogberg@mykopat.slu.se

‡Present address: The Swedish Species Information Centre, Swedish University of Agricultural Sciences, PO Box 7007, S-750 07 Uppsala, Sweden.

Lactarius mammosus Fr. is a common ectomycorrhizal (EM) basidiomycete distributed in coniferous forests in the boreal zone of the Northern Hemisphere forming mycorrhizas with Scots pine, *Pinus sylvestris* L. (Heilman-Clausen *et al*. 1998). This fungus may occur at all forest successional