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PERMANENT GENETIC RESOURCES NOTE

# Characterization of polymorphic microsatellite markers in the Dalmatian wall lizard *Podarcis melisellensis* (Squamata: Lacertidae)

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**Abstract**

**We describe polymerase chain reaction primers and amplification conditions for 13 highly polymorphic microsatellite DNA loci isolated from the Dalmatian wall lizard, *Podarcis melisellensis*. The number of alleles per locus ranged from 12 to 41, with levels of observed heterozygosity between 0.62 and 0.94. Most of these loci were successfully cross-amplified in the closely related species *P. sicula*, but levels of polymorphism were always lower.**

*Keywords*: cross-species amplification, Lacertidae, microsatellites, *Podarcis melisellensis*, *Podarcis sicula*

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The Dalmatian wall lizard, *Podarcis melisellensis* (Braun, 1877), is a small lizard (average snout-vent length ♂♂: 65.42 mm; ♀♀: 57.01; Brecko *et al*. 2008), endemic to the Adriatic coast and many of the islands in the Adriatic Sea. Individuals have one of three different ventral colours: bright white, yellow or orange (Huyghe *et al*. 2007). As part of an ongoing study about the origin and maintenance of this colour polymorphism, we developed a series of polymerase chain reaction (PCR) primers to screen microsatellite DNA loci which may be useful for assessing intraspecific population genetic variation, individual relatedness, and parentage. Through cross-species amplification in the closely related species *P. sicula* (Rafinesque, 1810), these microsatellites may also help to uncover alleged interspecific hybridization between both species.

Genomic DNA used for the isolation of microsatellite loci was extracted from a 2-mm tail tip tissue sample, using a standard protocol (QIAGEN DNA mini kit). DNA samples for the PCRs were extracted using a modified Chelex extraction protocol (adapted from R. J. Nelson, Institute of Ocean Sciences, personal communication; Small *et al*. 1998; Savannah River Ecology Laboratory Journal of Undergraduate Research 2005). A Chelex extraction buffer made with 10% Chelex (Bio-Rad), 0.1% SDS and 0.1% of 20 mg/mL Proteinase *K* was pipetted on the tissue samples, which were then incubated for 60 min at 65 °C and 15 min at 95 °C. Isolation of microsatellite loci was done by the VIB DNA core facility at the University of Antwerp. Briefly, the protocol used was as follows. A TOPO shotgun subcloning kit (Invitrogen) was used to construct a genomic library according to the manufacturer's instructions. Recombinant colonies were screened with $(CA)_{20}$, $(GA)_{20}$, $(TAA)_{10}$, and $(CAA)_{10}$ oligonucleotide probes that were $^{32}P$ radioactively end-labelled using a T4 polynucleotide kit (Invitrogen). Hybridization was performed overnight in a church buffer at 50 °C. Subsequent washing took place in 5× SSC, 15 min at room temperature, and 15 min at 50 °C. Positive clones were sequenced with M13 forward and M13 reverse vector primers. Sputnik (Espresso Software Development) was used to identify short tandem repeats, utilizing a recursive algorithm to search for 2–5 base repeated patterns of nucleotides. Using these sequences, we designed 20 primers using the program Primer3 (freely available at http://frodo.wi.mit.edu/). Forward primers were fluorescently labelled (6FAM dye: Sigma, and VIC, PET, NED dye: Applied Biosystems). Primers were tested for polymorphism on 100 individuals originating from a population of *P. melisellensis* of the island Lastovo (Croatia). *P. sicula* individuals ($N = 23$) for testing cross-amplification were caught on the nearby islet Pod Kopiste.

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**Table 1** Characterization of 13 polymorphic microsatellite loci isolated from *Podarcis melisellensis*

| Locus | Primer sequence (5′–3′) | Multiplex | $T_a$ (°C) | Repeat motif | Cloned allele size (bp) | $A$ | Size range (amplified allele) | $H_O$ | $H_E$ | HWE | $n$ | Null allele frequencies | GenBank Accession no. |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Pmeli-02 | F: 6FAM-TTCCAAGTCTGATTCACTCCAA<br>R: AGCTGCAAGCACCTAGCAAT | 1 | 60 | $(TG)_9(AG)_{10}$ | 727 | 40 | 139–223 | 0.78 | 0.96 | $P < 0.001$ | 97 | 0.090 | EU869334 |
| Pmeli-04 | F: VIC-GGGGAAGTTGCTGTGGTCTA<br>R: AGGCAGGCAAAATCCCTAAT | 1 | 60 | $(TC)_{24}$ | 816 | 28 | 134–228 | 0.85 | 0.95 | $P < 0.001$ | 94 | 0.051 | EU869323 |
| Pmeli-05 | F: VIC-AGCCATCTACGGCTCTTTCA<br>R: GGGACCCCAAATTGAGAAAA | 5 | 60 | $(TG)_8$ | 870 | 15 | 227–257 | 0.79 | 0.83 | $P = 0.14$ | 100 | N/A | EU869322 |
| Pmeli-07 | F: PET-AGTAACCCTTTCCGGCACTT<br>R: AGGATGAACACCTGCCAAAT | 1 | 60 | $(AC)_{18}$ | 1027 | 13 | 202–228 | 0.77 | 0.89 | $P = 0.001$ | 97 | 0.062 | EU869324 |
| Pmeli-08 | F: 6FAM-ATGCTCCCACCTGTCATCTC<br>R: TGATTGGCACCAGTGGAT TA | 3 | 59 | $(TG)_{18}(AG)_{22}$ | 623 | 22 | 158–224 | 0.91 | 0.93 | $P = 0.23$ | 99 | N/A | EU869325 |
| Pmeli-10 | F: NED-AGCTGTTTGGGGTTGAGTTG<br>R: TGTAGATGCAGCCAAAGCAG | 4 | 50 | $(TG)_{16}$ | 541 | 13 | 185–214 | 0.76 | 0.81 | $P = 0.11$ | 100 | N/A | EU869326 |
| Pmeli-11 | F: NED-ACCGAAAAACCCTTTCGTCT<br>R: CCTGCTTGGTGAAGGAAAAA | 3 | 59 | $(CA)_{14}$ | 623 | 12 | 175–205 | 0.73 | 0.74 | $P = 0.52$ | 99 | N/A | EU869327 |
| Pmeli-13 | F: VIC-AAATTAGCTCCCCCTTCTCC<br>R: GAGCCCGCCTAGACAAAAG | 2 | 57 | $(AC)_{22}$ | 254 | 17 | 186–211 | 0.86 | 0.84 | $P = 0.81$ | 100 | N/A | EU869328 |
| Pmeli-14 | F: 6FAM-GCTGGTGCTTTCTGGTCATT<br>R: CAGCAGTTTCCATGTTCCTG | 2 | 57 | $(AG)_{23}$ | 372 | 25 | 139–188 | 0.94 | 0.94 | $P = 0.54$ | 100 | N/A | EU869329 |
| Pmeli-15 | F: VIC-CCCTTTTGCACAACAGTTCA<br>R: CTGGCTTACAGTGGCAAACA | 3 | 59 | $(GT)_{18}$ | 506 | 14 | 118–149 | 0.76 | 0.79 | $P = 0.23$ | 100 | N/A | EU869330 |
| Pmeli-16 | F: 6FAM-GACGGGGGTTTAGGAAGAAG<br>R: GAGGCTCTGGGAAGATGTTG | 4 | 50 | $(AC)_8$ | 388 | 28 | 97–249 | 0.79 | 0.94 | $P < 0.001$ | 99 | 0.070 | EU869331 |
| Pmeli-18 | F: 6FAM-TGCGTTATTGTTGAGGCTGT<br>R: CCTGCACCTTGAATTGAGC | 5 | 60 | $(TC)_{17}$ | 1025 | 29 | 193–255 | 0.62 | 0.94 | $P < 0.001$ | 89 | 0.16 | EU869332 |
| Pmeli-19 | F: PET-TTGCGAGAGCTTTTTGAACC<br>R: GCAGATGACAAAGCAATAGCC | 2 | 57 | $(GC)_3(AC)_{10}$ | 625 | 41 | 157–241 | 0.77 | 0.97 | $P < 0.001$ | 96 | 0.15 | EU869333 |

$T_a$, annealing temperature; $A$, observed number of alleles; Size range in bp; $H_O$, observed heterozygosity; $H_E$, expected heterozygosity; HWE, Hardy-Weinberg probability; $n$, number of individuals.

Multiplex PCR amplifications were performed in a 12.5 μL reaction volume containing 1.5 μL of template DNA, 100× primer mix containing each primer at 0.02 μM, 4× QIAGEN multiplex PCR master mix (QIAGEN), and 3× RNase free $H_2O$. Amplification entailed an initial denaturation step of 15 min at 95 °C, followed by 30 cycles of 30 s denaturation at 95 °C, 90 s of annealing at loci-specific temperatures (Table 1), and 60 s of extension at 72 °C. After the last cycle, a final extension was done at 60 °C for 30 min. PCR conditions were optimized using a gradient thermal cycler (TGradient, Biometra), and after elimination of poorly amplifying loci, two or three primers with similar optimal temperatures were combined in five multiplex PCRs (Table 1). Finally, 13 loci were genotyped in 100 individuals of *P. melisellensis*. For fluorescent detection on an AB 3130 XL Genetic Analyser, PCR products were mixed with formamide and an internal size standard (500Liz, GeneScan). The size of the PCR products was determined using the GeneMapper version 4.0 software program (Applied Biosystems).

In *P. melisellensis*, all 13 loci were polymorphic with allele numbers ranging from 12 to 41. Observed ($H_O$) and expected ($H_E$) heterozygosities were estimated, and tests for Hardy–Weinberg equilibrium (HWE) were conducted with FSTAT version 2.9.3 (Goudet 1995). Null allele frequencies were obtained for loci not in HWE (Table 1) and evidence for genotypic linkage disequilibrium was tested using GenePop version 3.3 (Raymond & Rousset 1995). $H_O$ values ranged from 0.62 to 0.95, and were consistently lower than $H_E$ (range: 0.74–0.97). HWE was rejected for six loci after sequential Bonferroni correction (corrected alpha = 0.0038) (Table 1). The higher allelic variation at these loci (mean: 29.83 alleles) compared to HWE loci (mean: 16.86 alleles; $F_{1,11} = 9.04$; $P = 0.012$) suggests that insufficient sampling might explain the HWE deviations at these loci. The proportion of samples that did not amplify also differed between loci in HWE (mean: 0.43 samples) and loci not in HWE (mean: 4.67 samples; $F_{1,11} = 10.12$; $P = 0.009$), suggesting the presence of null alleles in the latter. However, null allele frequencies were low (all $P < 0.16$) for all non-HWE loci, and therefore, should not have a significant impact on, e.g. parentage analyses (Dakin & Avise 2004). There was no evidence for linkage disequilibrium among our loci (data not shown).

Cross-species amplification was attempted in 23 individuals of *P. sicula*. Although most loci yielded consistent amplification products (nine out of 13 loci) using the same PCR conditions described above, levels of polymorphism were very low compared to those of *P. melisellensis*, with allele numbers ranging from two to six only (Table 2). However, none of the loci showed HWE deviations after sequential Bonferroni correction. For several loci, *P. sicula* showed alleles not appearing in *P. melisellensis* (one to three alleles in six loci), suggesting that this primer set could be useful in other *Podarcis* species.

**Table 2** Cross-species amplification of microsatellite loci (*Podarcis sicula*). N/A no detectable PCR product

| Locus | *A* | Size range (amplified allele) | $H_O$ | $H_E$ | HWE | *n* |
|---|---|---|---|---|---|---|
| Pmeli-02 | 4 | 131–181 | 0.74 | 0.73 | $P = 0.63$ | 23 |
| Pmeli-04 | (0) | N/A | N/A | N/A | N/A | (23) |
| Pmeli-05 | (0) | N/A | N/A | N/A | N/A | (23) |
| Pmeli-07 | (0) | N/A | N/A | N/A | N/A | (23) |
| Pmeli-08 | 4 | 165–182 | 0.36 | 0.60 | $P = 0.02$ | 22 |
| Pmeli-10 | 2 | 196-198 | 0.04 | 0.04 | $P = 1.00$ | 23 |
| Pmeli-11 | (0) | N/A | N/A | N/A | N/A | (23) |
| Pmeli-13 | 2 | 193–195 | 0.52 | 0.43 | $P = 0.94$ | 23 |
| Pmeli-14 | 6 | 132–158 | 0.74 | 0.71 | $P = 0.70$ | 23 |
| Pmeli-15 | 5 | 111–147 | 0.52 | 0.59 | $P = 0.32$ | 23 |
| Pmeli-16 | 3 | 179–190 | 0.74 | 0.63 | $P = 0.92$ | 23 |
| Pmeli-18 | 5 | 213–224 | 0.65 | 0.65 | $P = 0.60$ | 23 |
| Pmeli-19 | 5 | 177–202 | 0.78 | 0.71 | $P = 0.85$ | 23 |

*A*, observed number of alleles ; Size range in bp; $H_O$, observed heterozygosity; $H_E$, expected heterozygosity; HWE, Hardy–Weinberg probability; *n*, number of individuals.

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