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TECHNICAL NOTE

Polymorphic microsatellite loci for the Balearic Island Lizard *Podarcis lilfordi* (Squamata: Lacertidae)

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Abstract Fifteen microsatellite loci are described for the lizard *Podarcis lilfordi* from the Balearic Islands. Loci were isolated from partial genomic libraries that had been enriched for AAAG and ACAG repeat sequence. All loci were highly polymorphic (six to 19 alleles) in a sample of 26 individuals from the island of Dragonera. Observed and expected heterozygosities ranged from 0.46 to 0.92 and 0.78 to 0.95, respectively. These loci will be used to study the evolutionary history of different forms, the occurrence of cross-islet introductions, and the validity of subspecific designations in this species.

Keywords Lizard · Lacertidae · *Podarcis lilfordi* · Microsatellite · Tetranucleotide · Balearic islands

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The endangered (CITES II listed) lizard species *Podarcis lilfordi* Günther 1874 is endemic to the Balearic Islands. Natural populations have become extinct on the main islands of Mallorca and Menorca but exist at very high densities on many of the tiny coastal rocks, islets and islands that surround them (Pérez-Mellado et al. 2008). This pattern is thought to have been mediated by human activities (e.g. introductions of other species) since man first began to inhabit the islands approximately 4,000–5,000 years ago (Bover et al. 2008 and references therein). Size, body proportions, scalation, colour and colour pattern vary considerably between populations and have formed the basis for the description of numerous subspecies by several different authors. Approximately 25 subspecies are currently recognized (Salvador 2009). Much of this variation seems to have originated during approximately the last 2.5 million years, and is at least partly due to cladogenesis following population isolation within different islands or regions (Brown et al. 2008). While attempts to determine the genetic "uniqueness" of each population using mitochondrial DNA (mtDNA) sequences have identified considerable fine-scale geographical structuring (Terrasa et al. 2009), a complete understanding of the significance of the intraspecific taxonomy, the occurrence of cross-islet introductions, and the evolutionary history of the different forms has been hampered by slow mtDNA fixation rates relative to recency of population vicariance. Markers with many alleles, such as microsatellite loci, have unprecedented ability to detect and describe genetic differences between populations at much smaller geographical scales and time intervals than has traditionally been possible using mtDNA (Queller et al. 1993). Microsatellite loci were developed for *P. lilfordi* with the aim of examining the evolutionary history of different forms, the occurrence of cross-islet introductions,

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and the significance of subspecific designations for this species.

Small-insert genomic libraries were constructed from size-selected fragments (250–1500 bp) of *P. lilfordi* genomic DNA (from a single individual from the island of Dragonera) that had been digested with *Mbo*I restriction enzyme, adapter ligated and enriched for AAAG and ACAG repeat sequence (for adapter sequence see Refseth et al. 1997). Hybridization capture of repeat sequence, recovery of enriched DNA by polymerase chain reaction (PCR), cloning and identification of microsatellite-containing clones by colony PCR were carried out as described in Bloor et al. (2006). Seventy-eight AAAG positive clones (identified from 624 colonies screened) and 12 ACAG positive clones (identified from 144 colonies screened) were sequenced on an ABI 3730 DNA Sequencer (Applied

Table 1 Summary data for microsatellite loci isolated from *Podarcis lilfordi*. Polymorphism data are based on 26 individuals of *P. lilfordi* from the island of Dragonera (see text for details)

Locus (accession no.)	Primer sequence (5′–3′)	Repeat array in clone	Size of cloned allele (bp)	T_a (°C)	N_a	Allele size range (bp)*	H_O (H_E)	*P*
Pli2	GATCAGGAAGGTAAAAGTGAGGA	$(GAAA)_7(GGAA)_{12}$	247	56	14	236–302	0.88	0.911
(HQ453273)	CCCCCTAAATAGTGACCCTAAG[a]						(0.90)	
Pli3	CATGAAGGGAGGCGATGTAT	$(GAAA)_{12}$	243	56	9	235–263	0.73	0.028
(HQ453274)	GATCCCATTCTGTCTTGGAA[d]						(0.80)	
Pli4	TCAGTTCATGCATAAGGTCCA	$(CTTT)_{14}$	382	56	19	344–406	0.88	0.396
(HQ453275)	TTCGGCATTTTTCTTCAGGT[a]						(0.95)	
Pli5	TGATATCCCGCTTTATCACTACC	$(CTTT)_{12}$	383	57	10	358–394	0.85	0.336
(HQ453276)	CTGACTGATACCAGAATTGTTGC[d]						(0.86)	
Pli6	ACATGTTGACGGGACTGGTA	$(CTTT)_{11}$	401	56	11	380–420	0.46	**0.001**
(HQ453277)	CCCTGGAGGAGCTATGAATG[c]						(0.78)	
Pli8	CTCTGGGAGAAATGCGTGT[d]	$(CTTT)_6(CCTT)(CTTT)_4$	135	56	13	125–179	0.65	0.116
(HQ453278)	GGTCCCTTCCAGCTCTACAA						(0.80)	
Pli9	AGCAGGCACACACAAGTAGC[b]	$(CTTT)_{11}$	385	55.5	11	365–431	0.85	0.806
(HQ453279)	TTGTAGGGTAAGGCCACACA						(0.86)	
Pli10	TGACTTGTAGGGCTGGCTTT[c]	$(CTTT)_6(CCTT)(CTTT)_4$	252	56	12	247–273	0.69	0.167
(HQ453280)	AGCTGTTTCTCAGCTGTGGTC						(0.84)	
Pli12	CAACAAAACCTTTGTAGAAGTTGG	Imperfect[1]	223	57	17	178–268	0.73	**0.001**
(HQ453281)	GATCTTCTGGGCCCTCTCTT[b]						(0.92)	
Pli16	GGGAGGTATCAGGCTGAGATT	$(CTTT)_{11}$	454	57	16	426–474	0.81	0.060
(HQ453282)	CTCCAGCAGGAAGGTAAACG[c]						(0.89)	
Pli17	GTAATTGCAGCCACCACACA[d]	$(GAAA)_{11}(GAAG)_2$	254	57	7	235–259	0.92	0.742
(HQ453283)	ATTTGTTGCATTTATAGTGCATTT						(0.82)	
Pli18	CAAGAATTGAGTTTGCAGTTCC	$(CTTT)_{13}$	133	55	9	119–151	0.81	0.344
(HQ453284)	TGTCTGACAGAATGTGCTTCTC[c]						(0.87)	
Pli21	CCATTATGACCTTGCTGGTG[d]	$(CTTT)_{12}$	141	57	17	128–196	0.58	**0.000**
(HQ453285)	GAACTCTGGTGGCCCACAT						(0.93)	
Pli22	TGCCTTTATAGCCACCCATAC	$(CTGT)_6$	110	55	6	107–123	0.65	0.888
(HQ453286)	CCATATCTGCCCATCTGGTT[d]						(0.71)	
Pli24	CCACAAGGACTCAGGCTCTC	$(CTGT)_7$	113	55	10	104–136	0.88	0.968
(HQ453287)	TCCCCCACTTAAGCATGTTC[b]						(0.87)	

P values (*P*) are given for tests of deviation from Hardy–Weinberg (H–W) equilibrium. Significant tests of deviation from H–W equilibrium after sequential Bonferroni correction are given in bold typeface

T_a annealing temperature (°C), N_a number of alleles. H_E and H_O correspond to expected and observed heterozygosities, respectively

[a] 6-FAM-labelled primer, [b] PET-labelled primer, [c] NED-labelled primer or [d] VIC-labelled primer

*Based on the GeneScan-500 (LIZ) internal size standard run on an ABI 3130 DNA Sequencer; 5′ ends of unlabelled primers were modified by addition of the sequence GTTTCTT to provide conditions for essentially full terminal nucleotide addition (see Brownstein et al. 1996), with the exception of loci Pli12, Pli17 and Pli21. [1]$(GAAA)_{13}(GAAAAGAAA)_2(GAAA)$

Biosystems; SECUGEN Sequencing Service). Fifty-three clones contained AAAG repeat sequence of 6 repeats or more and 5 clones contained ACAG repeat sequence of 6 repeats or more. Ten sequences were excluded because they corresponded to non-unique sequences.

Primer pairs were designed for 24 unique repeat-containing sequences using the program PRIMER3 (Rozen and Skaletsky 2000). All primer pairs were initially tested in single reactions. Amplifications that were consistent with polymorphic single-locus products of expected size were obtained for 15 loci (Table 1). One primer from each pair was fluorescently labelled (FAM, VIC, NED or PET) so that the corresponding PCR products could be distinguished from one another by size difference or by different fluorescent dyes (see Table 1). The sequencing of alleles revealed the presence of indels in the flanking sequence of three loci (Pli12, Pli17 and Pli21). This led to size variants that were inconsistent with the repeat unit length, and so new primers were designed to exclude these indels. Polymerase chain reactions (PCRs) were performed in 10-μL reaction volumes containing DyNAzyme 1 × reaction buffer (Finnzymes), 2.0 mM $MgCl_2$, 0.2 mM of each dNTP and 0.25 U DyNAzyme II DNA *Taq* Polymerase (Finnzymes) with 0.5 μM of each primer. Cycling conditions were 1 min at 94°C, then 34 cycles of 30 s at 94°C, 30 s at locus-specific annealing temperature (see Table 1) and 45 s at 72°C with a final step of 7 min at 72°C. Fluorescently labelled PCR products were run on an ABI 3130 DNA Sequencer (Applied Biosystems) with the GeneScan-500 (LIZ) internal size standard.

Twenty-six individuals of *P. lilfordi* from the island of Dragonera were typed to determine levels of polymorphism. Number of alleles per locus ranged from 6 to 19. Observed and expected heterozygosities ranged from 0.46 to 0.92 and 0.78 to 0.95, respectively. Tests of deviation from Hardy–Weinberg (H–W) equilibrium revealed significant ($P < 0.05$) departures from H–W expectations at three loci (Pli6, Pli12 and Pli21) after sequential Bonferroni correction (Rice 1989) (four loci were significant at the unadjusted significance level; Pli3, Pli6, Pli12 and Pli21). Pairwise comparisons of the 15 loci did not reveal evidence of linkage disequilibrium after sequential Bonferroni correction (seven of 105 tests were significant at the unadjusted significance level). All calculations were performed using the program GENEPOP version 3.4 (Raymond and Rousset 1995). The levels of polymorphism uncovered at these loci suggest that they should be useful for the study of population differentiation as well as the evolutionary histories of the different morphological forms present in this species.

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