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Isolation and characterization of sixteen polymorphic microsatellite loci in the Western Spadefoot, *Pelobates cultripes* (Anura: Pelobatidae) via 454 pyrosequencing

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Abstract

The Western Spadefoot, *Pelobates cultripes* (Anura, Pelobatidae), is endemic to the Iberian Peninsula and southeastern France, with isolated populations in the Atlantic coast of France. Its populations are fragmented and it is considered Near Threatened by the IUCN. Here we describe the development of sixteen polymorphic microsatellite loci in this species. Polymorphism was assessed in 95 individuals from five Iberian populations. The number of alleles and expected heterozygosity ranged from 3 to 14 and 0.20 to 0.76, respectively. Eight loci cross-amplified in the closely related and Endangered Moroccan Spadefoot toad, *Pelobates varaldii*. These markers will be useful to address questions about the ecology, population genetics and evolutionary history of *P. cultripes*, including information on effective population size, habitat use and dispersal patterns, which are essential for the efficient management of the fragmented populations characteristic of most of its range.

Keywords: microsatellites, amphibians, *Pelobates cultripes*, Iberian Peninsula, *Pelobates varaldii*, North Africa

Amphibians are the most endangered group of vertebrates, with nearly a third of the species threatened or extinct (IUCN et al. 2008). Currently their populations are declining in all regions of the world (Stuart et al. 2008). The most important causes of these declines are habitat destruction and fragmentation, infectious disease (chytridiomycosis) and climate change (Hof et al. 2011). In Europe, projection of species potential distributions under plausible future global change scenarios forecast an increase in suitable habitat for a great proportion of species, except in south-western Europe, where several species will experience a decline in the extent of suitable habitat (Araújo et al. 2006).

One of this species is the Western Spadefoot toad, *Pelobates cultripes* (Cuvier, 1829), which is distributed throughout most of the Iberian Peninsula, along the Mediterranean coast of France and in some disjunct areas in the French Atlantic coast (García-París et al. 2004; Loureiro et al. 2008; Duget and Melki 2003). Its populations are declining range-wide (Tejedo and Reques 2002) due to habitat loss and the negative impact of invasive species and consequently, the species is listed as Near Threatened by the IUCN (Beja et al. 2009).

Here we describe the isolation and characterization of sixteen polymorphic microsatellite loci in *P. cultripes* that will help address a suite of questions ranging from the ecology, demographics, population and landscape genetics, to the phylogeography of the species and provide valuable information for the management of its populations.

A genomic library was constructed at the Sequencing Genotyping Facility, Cornell Life Sciences Core Laboratory Center (CLC) (Andrés and Bogdanowicz 2011). It was developed from one tadpole (voucher: IMS1224, El Pedroso, Sevilla, Spain). Sequences containing microsatellites were scanned with iQDD 1.3 (Meglécz et al. 2010), and forty primer pairs flanking regions with microsatellite motifs were designed with a minimum length of flanking region of 20 bp and a range size between 90-320 bp, with an optimal melting temperature of 60ºC to facilitate multiplexing.

PCR reactions were performed in a total volume of 15 µl, including 25 ng of template DNA, 1x reaction buffer, 1.5 mM $MgCl_2$, 0.3 mM dNTP, 0.3 µM of each primer and 0.5U Taq polymerase. PCR cycling consisted of initial denaturation (95°C, 5 minutes), 35 cycles of denaturation (95°C, 45 seconds), annealing (60ºC, 45 seconds), and extension (72°C, 45 seconds), and a final extension (72°C, 10 minutes).

PCR products were visualized in 2.5 % agarose gels. Of the 40 pairs of primers tested, 24 showed unambiguous bands and were selected for further screening, although only sixteen amplified consistently in all samples. We

scored variation in 95 individuals from five populations distributed across the range of the species (Table 1). Additionally, we tested for cross-amplification in a sample of 17 individuals from three localities of the closely related, north African species *Pelobates varaldii*. This species is cataloged as Endangered by the IUCN due to its restricted and fragmented range and continuing decline in the extent and quality of habitat (Salvador et al. 2004).

Forward primers were labeled with fluorescent dyes (6-FAM, PET, NED, and VIC) for use in five multiplex reactions, which were designed with Multiplex Manager 1.2 (Holleley and Geerts 2009) and performed using Type-it Microsatellite PCR kits (Qiagen) (Table 2). All reactions were performed in a total volume of 15 µl, containing 7.5 µl of Master Mix, 1.2 µl of primer mix (0.2 µM of each primer), and 5.3 µl of RNase-free H_2O. Genotyping was performed on an ABI PRISM 3730 sequencer with the GeneScan 500 LIZ size standard (Applied Biosystems). Peaks were scored manually in GeneMapper 4.0 (Applied Biosystems).

The presence of null alleles, stuttering and large allele dropout in each population was tested using Microchecker 2.2.3 (Van Oosterhout et al. 2004). Allele dropouts or stuttering were detected in loci Pc4.7 (all populations with the exception of BOC) and Pc4.3 (all populations, except BOC and DON) and thus some caution is required if using these markers.

We estimated the number of alleles (Na), observed (Ho) and expected heterozygosity (He) for each locus and population with GenAlEx 6.5b5 (Peakall and Smouse 2006) and Genetix (Belkhir et al. 2000). The observed number of alleles ranged from 3 to 14 and their size from 119 to 300 bp. The average expected heterozygosity was 0.41 (range: 0.20–0.76) and the average observed heterozygosity, 0.44 (range: 0.20–0.73).

Deviation from Hardy-Weinberg equilibrium (HWE) and evidence of linkage disequilibrium (LD) were tested as implemented in Genepop version 4.2 (Rousset 2008). A sequential Bonferroni correction (Rice 1989) was applied to adjust for multiple comparisons. Deviations from HWE were detected in loci Pc4.3 (populations BOC and DON) and Pc4.7 (all populations, except BOC). Significant LD was detected in population DON for loci Pc3.1 and Pc4.5.

Eight out of the sixteen loci tested amplified consistently in *P. varaldii*, although only two of them (Pc3.2 and Pc3.9) were polymorphic (four alleles each).

These novel polymorphic microsatellite markers will add to those described by Van de Vliet et al. (2009) and provide valuable resources to address questions about the ecology, population genetics and evolutionary history of *P. cultripes*, including information on effective population size, habitat use and dispersal patterns, which are essential for the efficient management of the fragmented populations characteristic of most of its range.

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153 **Table 1**. Populations of *P. cultripes* screened for variation with the microsatellites developed in the present
154 study, including locality information, population code, number of samples per population (*n*), geographic
155 coordinates (latitude and longitude) and estimates of genetic diversity (number of alleles, observed and expected
156 heterozygosity).

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Locality	Code	n	Latitude	Longitude	N_A	H_O	H_E
Spain, Valencia, Sinarcas	SIN	19	39° 45' N	1° 14' W	3.5625	0.4605	0.4844
Spain, Madrid, Valdemanco	VAL	19	40° 51' N	3° 38' W	4.0000	0.4020	0.4519
Spain, Huelva, Doñana	DON	19	36° 59' N	6º 27' W	2.6875	0.4322	0.4484
Portugal, Setúbal, Boticos	BOT	19	38° 00' N	8° 29' W	5.1875	0.4281	0.5203
Portugal, Aveiro, Paramos	PAR	19	40° 58' N	8° 38' W	2.6250	0.3158	0.3026

Table 2. Characterization of 16 polymorphic microsatellite loci in the Western Spadefoot (*P. cultripes*), including locus designation, primer sequences, fluorescent dye, repeat motif, multiplex reaction, annealing temperature (ºC), size of amplified product (bp), number of individuals successfully genotyped (*n*), number of alleles (N_A), observed (H_o) and expected (H_e) heterozygosities, probability of deviation from Hardy–Weinberg equilibrium (P_{HW}) and GenBank accession number (to be added upon acceptance).

Locus	Primer sequence	Labeling dye	Repeat motif	Multiplex reaction	T^a (ºC)	Size range (bp)	*n*	N_A	H_o	H_e	P_{HW}	GenBank accession no.
Pc3.25	5' GCGTTGGTACACATTGCATC 3' 5' GGCAGCTGTGTAATCGACCT 3'	PET	$(GTT)_7$	Multiplex1	60	191 - 206	95	6	0,453	0,488	0,4348	
Pc4.1	5' CAAAATGTCCAGTTGGAGTGAG 3' 5' GGAATTTAAGGTGGAAGAGGG 3'	NED	$(TAGA)_5$	Multiplex1	60	151 - 209	95	14	0,484	0,485	0,0055	
Pc3.2	5' GCTTGTTTGACCTCGTCTCTG 3' 5' CCTCAATGACACCTCTCATGAAC 3'	6-FAM	$(TAA)_{12}$	Multiplex1	60	178 - 205	95	10	0,758	0,727	0,1313	
Pc3.9	5' GTGTTTCCTGCCAATTGCTT 3' 5' CGTTCACTGATGTCCCAATG 3'	VIC	$(TAA)_6$	Multiplex1	60	132 -144	95	5	0,316	0,282	0,2287	
Pc3.1	5' TTTGACTAGGGTCCATGCAA 3' 5' GGAAAGTTTTGGGTAAAGCG 3'	PET	$(TAT)_6$	Multiplex2	60	128 - 137	95	4	0,326	0,312	0,8734	
Pc4.4	5' GGCACACCAAAACACATTGA 3' 5' GACTGTTTATCTATCCATCCACCC 3'	NED	$(TGGA)_5$	Multiplex2	60	125 -145	95	6	0,379	0,403	0,4532	
Pc3.23	5' CCCTGTAAAGGGCATCATCT 3' 5' TAGGGTGGGAACATCAGGAG 3'	6-FAM	$(CCT)_5$	Multiplex2	60	178 - 203	95	5	0,253	0,302	0,5946	
Pc4.5	5' TTGCAACTGTATAGAGAGGTGATT 3' 5' TTTCTTTCCCTGTCCGAATG 3'	6-FAM	$(AGAA)_6$	Multiplex3	60	155 - 187	95	9	0,663	0,643	0,1354	
Pc3.3	5' TCATTGGGAGATCTGAAGCA 3' 5' TAACTGGCGTCCATCATTCA 3'	VIC	$(TGG)_6$	Multiplex3	60	207 - 225	95	3	0,200	0,196	0,2099	

Pc4.3	5' TTAGGGGAAATGCAAACCAA 3' 5' GAAATGCACCACCATTTTCC 3'	PET	$(GTTT)_7$	Multiplex3	60	157 - 178	93	9	0,300	0,466	<0,0001	
Pc3.7	5' ATACTCACCATCCCACCCAA 3' 5' TGGTAATCTGCATGTCCCCT 3'	PET	$(TAG)_{14}$	Multiplex4	60	125 - 158	95	11	0,547	0,530	0,5885	
Pc4.11	5' TCTGCTTGGCCCCTATAGTC 3' 5' TGCAACAAGTTTGGACCAATTA 3'	NED	$(GATA)_5$	Multiplex4	60	119 - 139	93	6	0,242	0,291	0,2769	
Pc4.9	5' TGTTCACACCGTAGACAGCA 3' 5' CGCAATGTACAATTGACAACAG 3'	VIC	$(ATTT)_5$	Multiplex4	60	133 - 149	94	5	0,628	0,580	0,5935	
Pc3.24	5' AATCCCACCATCGTCAATGT 3' 5' TCCTACTACCCCAAGGCTGA 3'	PET	$(CCA)_6$	Multiplex5	60	224 - 239	95	4	0,200	0,198	0,0707	
Pc4.7	5' TTCAATCTGGGCTGAAGAGG 3' 5' AGGCCAAACGACTGAATTTG 3'	6-FAM	$(GATA)_7$	Multiplex5	60	159 - 207	94	11	0,287	0,678	0,2138	
Pc3.4	5' AGCATACTCCCTACTTGAACCA 3' 5' AGCTAATTCCCCATTCGGTT 3'	6-FAM	$(ACA)_6$	Multiplex5	60	288 - 300	94	5	0,488	0,483	<0,0001	