Conserv Genet (2009) 10:993–996
DOI 10.1007/s10592-008-9670-6

TECHNICAL NOTE

Development and characterization of highly polymorphic microsatellite loci for the Western Spadefoot toad, *Pelobates cultripes*

Mirjam S. van de Vliet · Onno E. Diekmann · Ester T. A. Serrão · Pedro Beja

Received: 11 July 2008 / Accepted: 13 August 2008 / Published online: 29 August 2008
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Abstract Nine highly polymorphic microsatellite markers were isolated and characterized for the Western Spadefoot, *Pelobates cultripes*. Remarkably, for this amphibian species high numbers of microsatellites were found as part of larger repeat containing regions, making primer design difficult. For nine loci, primers were designed successfully and genotyping of individuals was reliable and consistent. Number of alleles and heterozygosity for these loci ranged from 9 to 34 and from 0.72 to 0.94, respectively. The high levels of polymorphism revealed by our developed loci should provide insight into population genetic structure and levels of dispersal for this typical Mediterranean temporary pond-breeding amphibian.

Keywords *Pelobates cultripes* · Western Spadefoot · *Pelobates fuscus* · Microsatellites · Cross-species amplification · Temporary ponds · Amphibian conservation genetics

The Western or Iberian Spadefoot toad, *Pelobates cultripes* is an amphibian species which typically breeds in temporary ponds in Mediterranean farmland. Next to some other unique (amphibian) species their life-history is highly adapted to these ephemeral waters with unpredictable flooding time and length of hydroperiod by e.g. enormous flexibility in breeding date and age of metamorphosis (Jacob et al. 2003). Mediterranean temporary ponds are unfortunately poorly understood and highly endangered; suffering widespread degradation and loss due to agricultural intensification and increasing urban use (Oldham 1999; Stoate et al. 2001). Agricultural intensification is one of the main causes of habitat loss influencing amphibian abundance (Beja and Alcazar 2003). An assessment of metapopulation structure would be useful to clarify the influence of landscape features and alterations on population persistence. Recently, the presence of a spatial metapopulation structure in small vertebrates inhabiting Mediterranean farmland has been demonstrated (Pita et al. 2007) and is possibly widespread, but for amphibians there is still little evidence and more information of their genetic population structure is needed. Amphibian species vary widely in dispersal and colonization ability, risk of local extinction, and sensitivity to habitat fragmentation, making it desirable to conduct correlative studies of different amphibian species (Marsh and Trenham 2001; Brede and Beebee 2004). Next to the Parsley frog (*Pelodytes punctatus*), for which microsatellites have been developed (Van de Vliet et al. 2008), the Western Spadefoot is an appropriate second model species for investigating metapopulation structures in Mediterranean temporary ponds under different land use regimes. The observed patterns can help to identify possible threats and migration barriers influencing population structure and survival; information which can help in proposing accurate conservation and management actions for temporary ponds in the area.

In this study nine highly variable microsatellite loci are developed for the Western Spadefoot toad following

M. S. van de Vliet (✉) · O. E. Diekmann · E. T. A. Serrão
CCMAR-CIMAR, F.C.M.A, University of Algarve,
Gambelas, P-8005-139 Faro, Portugal
e-mail: mvliet@ualg.pt

P. Beja
ERENA, Ordenamento e Gestão de Recursos Naturais Lda,
Rua Robalo Gouveia, 1-1A, 1900-392 Lisboa, Portugal

P. Beja
CIBIO – Centro de Investigação em Biodiversidade e Recursos
Genéticos, Campus Agrário de Vairão, 4485-66 Vairão, Portugal

Gautschi et al. (2000). Genomic DNA libraries were constructed using DNA from four tadpoles collected in the south-west of Portugal using a standard phenol-chloroform extraction protocol (Sambrook et al. 1989). Fragments containing repeat rich regions were hybridized with five 5′-biotinylated oligonucleotide repeat probes: $(CA)_{15}$, $(GA)_{15}$, $(CAC)_{10}$, $(CAG)_{10}$ and $(ATAG)_8$ and attached to streptavidin-coated magnetic beads (Dynabeads M-280 Streptavidin, DYNAL biotech, Hamburg). Library construction and screening methods are described in more detail elsewhere (Van de Vliet et al. 2008).

Approximately 62 clones of the 520 totally screened clones (12%) gave a strong positive hybridization signal. Of these, 24, 6 and 32 inserts from di-, tri- and tetranucleotide libraries, respectively, were sequenced in an ABI prism 3130XL capillary sequencer. All inserts sequenced contained repeats, but compared with microsatellite regions found in the Parsley frog (Van de Vliet et al. 2008), most microsatellites were short and found in long compound repeat regions (65% of all sequenced inserts). These repeat containing regions could be more than 500 bp long, which made primer design impossible and which would make genotyping of these loci unreliable. As found when developing a library for the Parsley frog $(ATAG)_n$ microsatellite isolation seemed most successful; 50% of the sequenced positives from the tetranucleotide library contained an $(ATAG)_{>10}$ repeat. For dinucleotide repeats (repeat number >10), isolation success was about 38% and again for this species also no long trinucleotide repeat motifs were found. Library construction of the Western Spadefoot (*Pelobates cultripes*) and the Parsley frog (*Pelodytes punctatus*) were developed simultaneously using the same protocol.

Primers were designed for inserts containing microsatellites with more than 10 single repeat type units (preferably with one repeat type in the fragment) and with flanking regions sufficiently large to allow primer development. A total of 12 primer pairs (4 and 8 for dinucleotide and tetranucleotide loci, respectively) were designed using mPRIMER3 software (http://bioinfo.ebc.ee/mprimer3/) and by eye. Primer pairs were tested in PCRs with DNA from 44 individuals of *Pelobates cultripes* from different locations along the south-west coast of Portugal. Those which yielded strong, single-band products after optimization of PCR conditions were then screened for polymorphism on polyacrylamide gels. PCR amplifications were performed in a 10 μl reaction volume containing approximately 20 ng DNA, 1 μM primer and 0.1 μM of fluorescently labelled reverse primer, 2.0 mM $MgCl_2$, 5X Go*Tag* Flexi buffer (Promega), 0.2 mM of each dNTP and 0.75 U Go*Tag* DNA Polymerase (Promega). The touchdown PCR program for 3 loci held 2 cycles of 30 s at 94°C, 30 s at 62°C, 30 s at 72°C, followed by 14 cycles of 0.5°C touchdown: 15 s at 94°C, 15 s at 62–55°C, 15 s at 72°C, followed by 24 cycles of 15 s at 94°C, 15 s at 55°C, 15 s at 72°C. For the other 6 remaining loci we used the following PCR program: 30 cycles with 95°C for 40 s, locus specific annealing temperature (Table 1) for 40 s, and 72°C for 40 s. For all PCR reactions we started with a denaturation step of 94°C for 5 min and the last cycle was followed by a 7 min extension at 72°C. Fragment size was determined on an ABI prism 3130XL capillary sequencer using LIZ 500 (Applied Biosystems) as an internal size standard. Allelic diversity, observed (H_O) and expected heterozygosity (H_E), deviation from Hardy–Weinberg equilibrium and linkage disequilibrium were calculated using GENETIX ver. 4.04 (Belkhir et al. 2000).

In order to prevent misinterpretation of allele banding pattern which would give an individual a wrong multilocus genotype, we selected loci which only give clear and consistent allele banding patterns with little ‘stutter’. This resulted in 9 microsatellites which amplify successfully according to our criteria. All 9 loci are highly polymorphic, showing also high allelic diversity between individuals within a population. Table 1 summarizes the characteristics of the primer pairs of the 9 loci. Number of alleles ranged from 9 to 34 and observed heterozygosity ranged from 0.72 to 0.94 and did not deviate significantly from expected values. No linkage disequilibrium was detected among any pair of loci. There was no evidence for allelic dropout or for null alleles using software MICRO-CHECKER (van Oosterhout et al. 2003).

Because the developed microsatellite markers might also be useful for population genetic studies for a related species which are also under the threat of landscape fragmentation and loss, six individuals of the Common Spadefoot *Pelobates fuscus* were tested using the same PCR conditions. The individuals were sampled from one location (Valthe; Drenthe) in The Netherlands. Only loci Pcu 1, Pcu 3 and Pcu 9 produced clear and consistent amplification products in the expected size range. All six individuals were homozygous for these three loci, but this result is maybe not surprising when using a low number of individuals from one location. Also genetic diversity in the Dutch populations of *P. fuscus* is probably low because populations are isolated and rapidly declining (Stichting RAVON, the Netherlands). The nine highly variable loci presented here will be a valuable tool to address questions at the level of metapopulations and further conservation genetic issues for *P. cultripes*.

Table 1 Nine polymorphic microsatellite loci for the species *Pelobates cultripes*

Locus	Primer sequence (5′–3′)	Repeat structure	No. of alleles	Size range (bp)	T_a (°C)	H_E	H_O
Pcu 1	AGGTAAACCCCATTCTAGTTACCGTT	$(TCTA)_{11}$	12	126–170	57	0.76	0.65
	GGATTGTAAACCTGCAAAATACATGCC						
Pcu 2	GGACTGTAACTGCCGTACCATC	$(AC)_{25}$	16	129–169	Tdown62-55	0.83	0.74
	CATTGATTCTCTTAGCCTCTGCGT						
Pcu 3	GAATGTGCATGATTAGAATGTGTCATTTATTGCT	$(ATCT)_{11}$	10	230–282	57	0.83	0.74
	TGACGGAACGAGAGAGACAGGG						
Pcu 4	ACCAAAGCAGAACAGTACTTACTTACAC	$(ATAG)_3ACAG(ATAG)_{11}$	10	125–167	60	0.72	0.74
	AACAATAGAAACAGAAAGTCCACGCT						
Pcu 5	AGCATCTAACCTCACCACTCC	$(TCTA)_8(TCTG)_7(TCTA)_3(TCTG)_2(TCTA)_{16}$	23	118–360	60	0.81	0.74
	AATCAGATGAAACATCAGGCACAA	$(TCTG)(TCTA)(TCTG)(TCTA)_6$					
Pcu 6	CATTGTCACTTAGGGCAGGGG	$(AGAC)_5(AGAT)(AGAC)_3AGGC(AGAC)_2$	21	192–290	60	0.89	0.87
	AGCACTGAAATACCTATTGATCTACCT	$AGGC(AGAT)(AGAC)_4(AGAT)_{17}$					
Pcu 7	ACTGAACTGCTTGTGCTTATTTC	$(AC)_3(AGAT)_{13}(AGAC)_3$	18	182–250	57	0.91	0.96
	GACAGATTGTCAATGTCTGTCT						
Pcu 8	ACATCTCCTGACCTCAAATCTTC	$(CTAT)_{18}CTTT(CTAT)_{11}CCTGT(CTAT)_{11}$	34	142–316	Tdown62-55	0.94	0.91
	TAGACAGACAGACAGAAAGACAG						
Pcu 9	TGAGACTTCCCACATCATCAAGCA	$(TC)_6CC(TC)_{12}$	9	95–139	Tdown62-55	0.77	0.74
	TCACCTATTCACACCTTCCACGAG						

Allelic data and genetic variation were derived from 44 individuals of *Pelobates cultripes* from different locations along the south-west coast of Portugal. T_a, locus-specific annealing temperature (touchdown program: start temperature–end temperature); H_O, observed heterozygosity; H_E, expected heterozygosity. Size range refers to the PCR product size at each locus. The characteristics of the repeat are based on the sequenced clones (GenBank Accession numbers EU881077–EU881085)

Acknowledgements This work was supported by Fundação para a Ciência e Tecnologia (Portugal) with support from FEDER, through research project PTDC/BIA-BDE/68730/2006, and SFRH/BD/24064/2005 PhD grant (FCT, ESF). We are very grateful to Margarida Machado, João Reis and Luís Cancela da Fonseca from the University of Algarve and Wiepke van de Vliet for their field assistance and their help in collecting samples. We also would like to thank Nuno Ferrand (CIBIO, Portugal) for his helpful comments and suggestions and I'm very grateful to Wilbert Bosman (Stichting RAVON, the Netherlands) for providing us with samples of *Pelobates fuscus*.

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