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Isolation of highly polymorphic microsatellite loci for a species with a large genome size: sharp-ribbed salamander (*Pleurodeles waltl*)

MIRJAM S. VAN DE VLIET*, ONNO E. DIEKMANN*, ESTER A. SERRÃO* and PEDRO BEJA†‡
*CCMAR-CIMAR, F.C.M.A- University of Algarve, Gambelas, P-8005-139 Faro, Portugal, †ERENA, Ordenamento e Gestão de Recursos Naturais Lda, Rua Robalo Gouveia, 1-1A, 1900-392 Lisboa, Portugal, ‡CIBIO, Centro de Investigação em Biodiversidade e Recursos Genéticos, Campus Agrário de Vairão, 4485-66 Vairão, Portugal

Abstract

Fourteen highly polymorphic microsatellite markers were developed and characterized for the sharp-ribbed salamander, *Pleurodeles waltl*. Isolating microsatellites with more than 12 single repeat type units was only successful for a tetranucleotide repeat (ATAG). Compared to microsatellite libraries constructed simultaneously for two anuran amphibian species, a greater number of primer pairs designed for *P. waltl* had to be discarded, due to consistent amplification problems. Low amplification success rate for *P. waltl* may be due to its larger genome size. Consequently, to avoid nonspecific binding and to increase amplification success, polymerase chain reaction programmes with touchdown cycles were used. For 14 microsatellite markers, amplification was successful and consistent with number of alleles and expected heterozygosity ranging from seven to 22 and from 0.79 to 0.94, respectively. All 14 microsatellite markers will be extremely useful for metapopulation studies of this unique amphibian species.

Keywords: amplification success, large genome size, microsatellites, *Pleurodeles waltl*, sharp-ribbed salamander

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Amphibians are proving to be model organisms in metapopulation dynamics (Rowe *et al*. 2000; Jehle & Arentzen 2002). Metapopulation approaches are favoured in studies dealing with habitat loss and fragmentation (Hanski 1998). Microsatellite-based analyses appear particularly powerful for identifying metapopulation structure, source–sink dynamics and barriers to migration (Rowe *et al*. 2000; Jehle & Arentzen 2002). Unfortunately, isolation success and availability of useful microsatellites for amphibians, particularly Caudata, is not high (Jehle & Arentzen 2002; Zane *et al*. 2002). Additionally, genome size varies enormously in amphibians (Gregory *et al*. 2007), which may affect polymerase chain reaction (PCR) amplification success (Garner 2002), especially for Caudata which are characterized by large genomes [Animal Genome Size (AGS) Database; Gregory 2001]. The sharp-ribbed salamander, *Pleurodeles waltl*, is an example of a caudate amphibian with large genome size. This species breeds preferably in temporary ponds in Mediterranean agricultural landscapes (Beja & Alcazar 2003). These ephemeral waters are highly vulnerable to human activities and are further threatened by agriculture intensification (e.g. Oldham 1999). Understanding metapopulation structure is needed to evaluate influences of landscape fragmentation and loss on amphibian population dynamics (Marsh & Trenham 2001).

The development of 14 microsatellite markers is here described following Gautschi *et al*. (2000). Genomic DNA libraries were constructed using DNA extracted from four tadpoles and one adult tailtip using standard phenol–chloroform extractions (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,

Correspondence: Mirjam van de Vliet, Fax: 00351 289800069; E-mail: mvliet@ualg.pt

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DYNAL Biotech). Simultaneously, using identical protocols, libraries were developed for two other amphibian species, the parsley frog (*Pelodytes punctatus,* Van de Vliet *et al*. 2008a) and the western spadefoot (*Pelobates cultripes,* Van de Vliet *et al*. 2008b).

For *P. waltl*, 112 clones of the total 720 screened (15%) gave strong hybridization signal. A total of 22, 18 and 72 inserts from di-, tri- and tetranucleotide libraries, respectively, were sequenced in an ABI PRISM 3130xl capillary sequencer. All inserts sequenced contained repeats, but only inserts enriched for tetranucleotide repeats contained long (> 10) uninterrupted microsatellites (89%). For inserts enriched for di- and trinucleotide repeats, we found only compound repeats, often forming long (> 500 bp) repeat-containing regions. The higher success of isolating $(ATAG)_{n>10}$ microsatellites compared to di- and trinucleotide repeats was also found in the libraries for the other two amphibian species (Van de Vliet *et al*. 2008a, b). For *P. cultripes,* most dinucleotide microsatellites were also compound repeats (65% of all sequenced inserts). Although dinucleotide repeats are the most abundant microsatellites reported for vertebrates (Tóth *et al*. 2000), for these and other (Arens *et al*. 2000) amphibian species they seem to be more compound and present in repeat rich regions. Microsatellites are ubiquitously present in vertebrate genomes, but frequencies of different repeat motifs and numbers of repeats in repetitive regions are still poorly understood.

Primers were designed for inserts containing microsatellites with more than 12 single repeat units (preferably with only one repeat type) and with flanking regions sufficiently large to allow primer development. A total of 35 primer pairs (1 dinucleotide locus and 34 tetranucleotide loci) were designed using mPrimer3 (http://bioinfo.ebc.ee/mprimer3/). Unfortunately, the single dinucleotide repeat microsatellite and also 20 tetranucleotide microsatellites had to be discarded (designed primer fail rate was 60%). Even after using more stringent PCR conditions and redesigning primer pairs, they only produced multiple bands or gave a smear after PCR amplification. This is in contrast to primers developed simultaneously for *P. punctatus* and *P. cultripes* (designed primer fail rates were 22% and 25%, respectively). For these two amphibian species amplifications were mostly strong, single-band products using common PCR conditions differing only in annealing temperatures for optimal amplification. PCR problems for *P. waltl* can possibly be explained by their large genome size; average *C*-value is 20.44 pg (AGS Database; Gregory 2001). For *P. punctatus* and *Pelobates* sp., the average genome sizes are much smaller (Gregory 2001): *C*-value is 2.44 pg for *P. punctatus* and approximately 4.39 pg for *P. cultripes* (average *C*-value of the nearest phylogenetic relatives *Pelobates fuscus* and *Pelobates syriacus*). These values reveal a positive correlation between genome size and primer amplification success, as shown previously by Garner (2002). Multiple PCR products found for a large set of primers developed for *P. waltl* could also be explained by sequence duplications within the genome, but we have no sequence data to support this.

Only 14 primer pairs gave strong, single-band products on an agarose gel after optimizing PCR conditions. For 13 of the 14 primer pairs, this could be achieved by using a touchdown programme to prevent nonspecific binding. Screening for polymorphism was performed by PCR in 10-μL reaction volumes containing approximately 20 ng DNA, 1 μM primer and 0.1 μM of fluorescently labelled reverse primer, 2.0 mM $MgCl_2$, 5× Go*Tag* Flexi buffer (Promega), 0.2 mM of each dNTP and 0.75 U Go*Tag* DNA Polymerase (Promega). The touchdown PCR programme held two cycles of 30 s at 94 °C, 30 s at a locus-specific annealing start temperature (Table 1), 30 s at 72 °C, followed by 10 or 14 cycles of touchdown: 15 s at 94 °C, 15 s at a locus-specific start temperature (Table 1) reduced by 0.5 °C each subsequent cycle, 15 s at 72 °C, followed by 24 or 30 cycles of 15 s at 94 °C, 15 s at locus-specific annealing end temperature (Table 1), 15 s at 72 °C. For locus Ppl 4, a simpler programme could be used: 30 cycles with 95 °C for 40 s, 55 °C for 40 s, and 72 °C for 40 s. For all PCRs, 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. All PCRs were performed on a GeneAmp 2700 thermocycler (Applied Biosystems). Fragment size was determined on an ABI PRISM 3130xl capillary sequencer using LIZ 500 (Applied Biosystems) as internal size standard. Allelic diversity, observed and expected heterozygosities, deviation from Hardy–Weinberg equilibrium and linkage disequilibrium were estimated using Genetix 4.04 (Belkhir *et al*. 2000).

The results obtained for the 14 remaining loci were very promising for further population genetic analyses. We characterized 38 individuals from different ponds in southwest Portugal. The spatial scale of an interbreeding population is unknown but there were no differences in tests of deviations from Hardy–Weinberg equilibrium using only 20 individuals from a group of nearby ponds or using all 38 individuals from all ponds. All loci were highly polymorphic: number of alleles ranged from seven to 22 and expected heterozygosity ranged from 0.79 to 0.94 (Table 1). Locus Ppl 10 showed significant deviation from Hardy–Weinberg expectations ($P < 0.05$), which may have been due to null alleles (as determined using the software Micro-Checker, van Oosterhout *et al*. 2003). Linkage disequilibrium was detected in one locus pair: Ppl 12/Ppl 13 after Bonferroni correction. Because we are dealing with a species with a large genome size, the relative amounts of target DNA and of available primers for amplifying target DNA could be reduced (Garner 2002), possibly increasing allelic dropout. Also possible nonspecific binding of primers (Garner 2002) and possible sequence duplications within the genome might cause amplification of nontarget DNA; consequently,

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Table 1 Fourteen polymorphic microsatellite loci for the species *Pleurodeles waltl*

Locus	Primer sequence (5′–3′)	Repeat structure	No. of alleles	Size range (bp)	T_a (°C) start-end	H_E	H_O
Ppl 1	TGCTGATAGGTGAAAAGTCGGGTTGG GGATACAGGCGGGCAGAACGAG	$(TATC)_{18}$	10	318–362	62–57*	0.86	0.81
Ppl 2	GTAGACAGTGGGGATAGAGAG AAATAGGAGGTCGTAGCCATC	$(AGAT)_{23}$	16	105–171	62–55*	0.91	0.74
Ppl 3	GGGTTTGCCACCCTGCCAT CATCCGCATTGGCTCTTTGC	$(AGAT)_{17}$	22	179–315	62–57	0.94	0.86
Ppl 4	TTGCTTTGTATTGGTAAACTTGGG ACTGCAATATGTAGCTTATCTTAAAAA	$(ATCT)_{21}ATTT(ATCT)_{16}$	19	127–229	55	0.92	0.84
Ppl 5	ATGGAGCCAGCACCTTTAACACC GAATGCACGCGCCAACATGAA	$(TCTA)_{16}$	15	151–219	62–59*	0.87	0.78
Ppl 6	GCTAAGGCCCAGAAAGAACAGGCA TCTTACCGTGTGGCGTTCCCAAG	$(TAGA)_{16}$	10	180–236	62–57	0.85	0.87
Ppl 7	CCTTTACCACTCAAGGTAAGTACAG GACACACAAATACATGCACATAGAG	$(TATC)_{17}TGTC(TATC)_2$	7	157–181	59–52	0.79	0.73
Ppl 8	AGCCAAAGCGACGACGAAAGAGTG AATAACTCCAGCTGTGGCAGCAAAGG	$(AGAT)_{16}$	11	187–227	62–57	0.88	0.76
Ppl 9	AATAACTCCAGCTGTGGCAGCAAAGG GGCAGATTGGATGGGTGAGTGGA	$(ATCT)_{23}$	11	230–270	62–55*	0.87	0.92
Ppl 10	TGAGACGCAAGGTGGGTTGG AACGTCCCTGTAACCTTTGTTAGTTT	$(TCTA)_{22}$	16	142–336	62–55*	0.87	0.68
Ppl 11	GACTAACTGTTGCATGTCATATC AGGGAGATAGCTAAAGGCTG	$(TATC)_{27}$	20	122–196	62–55*	0.92	0.82
Ppl 12	CTGTAGAGAGGTTTGGGGCTAACA GGTGCATGGCATCAAGGAGG	$(ATCT)_{19}$	13	152–220	62–55*	0.89	0.84
Ppl 13	AACATGCCACCCCAGTTTTG CCTACTTATAACATCCTTGTAACCTTTG	$(TAGA)_{19}$	18	163–235	62–55*	0.89	0.87
Ppl 14	CCGTCCTCTGCTTCAATGGC AAGAGAGACAGGTAGAGGTGTTGATT	$(ATCT)_{20}$	14	297–347	62–55	0.91	0.89

Allelic data and genetic variation were derived from 38 individuals of *Pleurodeles waltl* from different ponds in southwest Portugal. T_a, locus-specific annealing temperature (touchdown programme: start temperature – end temperature); *longer programme with 30 cycles; 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 nos EU825704–EU825717).

genotyping error rate could be high for this species. To accurately estimate genotyping error rate, re-extraction and genotyping of a randomly selected replicate sample of 5–10% is recommended (Pompanon *et al*. 2005). Two randomly selected samples were re-extracted and no such errors (differences in their multilocus genotypes) were found.

PCR amplification problems, possibly due to the large genome size of the species, were improved by using PCR programmes with touchdown cycles. The high numbers of alleles and heterozygosity per locus will make these microsatellite markers highly valuable for metapopulation genetic studies.

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