

PRIMER NOTE

Identification and characterization of microsatellite markers in Hermann's tortoise (*Testudo hermanni*, Testudinidae)

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Abstract

The isolation and characterization of six polymorphic loci from a *Testudo hermanni* genomic library is reported. Enrichment was performed for AC but four of the characterized microsatellites present also an additional motif. Variability was tested on populations of the two recognized subspecies, *Testudo hermanni hermanni* and *Testudo hermanni boettgeri*. For one locus, a size range specific for the subspecies *T. h. hermanni* was observed. These are the first primers identified directly in the genome of *T. hermanni*

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The Hermann's tortoise (*Testudo hermanni* GMELIN, 1789) is widespread in the Mediterranean regions from Catalonia to the Balkans, up to European Turkey. Two subspecies are recognized, *Testudo hermanni hermanni* and *Testudo hermanni boettgeri*, inhabiting the western (Spain, France, and Italy) and the eastern (all the other countries) parts of the distribution range, respectively (Bour 1997). Recent urbanization severely fragmented in small patches the suitable habitats of this species, affecting its distribution and its geographical structure. Presently, the species is seriously threatened in certain parts of its range.

Here we report a set of novel polymorphic microsatellites that will be useful to describe the levels of genetic variability and divergence within and between populations, thus providing an initial genetic framework useful to identify conservation priorities and develop management strategies.

Genomic DNA was extracted from total blood (10–100 µL) using DNeasy Blood and Tissue Extraction Kit (QIAGEN). All polymerase chain reactions (PCRs) were performed on GeneAmp 9700 or GeneAmp 2700 thermal cycler (Perkin-Elmer).

A partial genomic library was constructed after enrichment with a biotin-labelled dinucleotide probe, adapting the FIASCO procedure (Zane *et al.* 2002a) to the following experimental conditions.

About 200 ng of genomic DNA were digested with *MseI* restriction enzyme and simultaneously ligated to *MseI* adaptor (Vos *et al.* 1995), for 3 h at 37 °C in a total volume of 25 µL containing: 1X OnePhorAll buffer (Pharmacia), DTT 5 mM, 0.05 mg/mL of BSA, 2.5 units of *MseI* (New England Biolabs), adaptor 1 µM, ATP 200 µM, 1 unit of T4 DNA ligase (Amersham-Pharmacia Biotech). The product of the digestion-ligation reaction was tenfold diluted and 5 µL were used as template in a PCR as in Zane *et al.* (2002b). For the enrichment, 80 pmol of a biotinylated dinucleotide probe (AC)₁₇ were mixed with 400 ng of the previous amplification product in 100 µL of SSC 4.2X, SDS 0.07%. After a 3 min denaturation at 95 °C, annealing was done at room temperature. After hybridization 300 µL of TEN (10 mM Tris-HCl, 1 mM EDTA, 100 mM NaCl, pH = 7.5) were added. The biotinylated fragments-probe complexes were recovered using one-milligram-streptavidin-coated beads (Streptavidin Magnetic Particles, Boehringer-Mannheim). After 30 min of hybridization at room temperature, beads-probe-DNA complex was separated by a magnetic field from the hybridization buffer. Three non-stringency, three stringency washes and the following elution were performed as in Zane *et al.* (2002b). After isopropanol precipitation 1 µL of this enriched solution was used as template in a 30 cycles PCR amplification using the above primer and the conditions previously described. PCR products were cloned in pGEM-T Easy Vector System I (Promega). About 200 recombinant colonies were amplified

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by PCR with universal M13 primers and screened by dot blot hybridization using the Dig Nucleic Acid Detection Kit in which the antidigoxigenin alkaline phosphatase conjugate was replaced by an antibiotin analogous (Alkaline Phosphatase Coniugated Anti-Biotin, Sigma). For hybridization and immunological detection the protocol provided by the previously mentioned kit was followed. Among the ones positive to the hybridization, 30 clones were sequenced and 24 contained a microsatellite motif.

Specific primers pairs were designed for 10 loci and all of them gave an amplification product of the expected size from *T. hermanni* genomic DNA. For six loci, one of the two primers was labelled with fluorescent dyes for further detection on ABI 310 Applied Biosystems (Table 1).

PCR amplifications were performed in 25 μ L containing: *Taq* buffer 1X (Polymed), $MgCl_2$ 1.5 mM, 0.6 μ M of each primer, 200 μ M dNTP's, 1 unit of *Taq*, and about 50 ng of genomic DNA. Thermal cycle was set as follows: 2 min of initial denaturation at 94 °C followed by 35 cycles of denaturation at 94 °C for 30 s, 1 min of annealing (Table 1), 1 min of elongation at 72 °C.

The variability of the six loci was investigated on 29 *T. h. hermanni* and 44 *T. h. boettgeri* individuals (Table 1). All loci resulted to be polymorphic with number of alleles ranging from two to 10.

No evidence for linkage disequilibrium among pairwise loci was detected by likelihood ratio test with 100.000 permutations, implemented in ARLEQUIN (Schneider *et al.* 2000). Significant departures from Hardy–Weinberg equilibrium were detected at loci Test 10 (both populations), Test 21 (*T. h. hermanni*) and Test 56 (*T. h. boettgeri*), following the procedure described in Guo & Thompson (1992) and implemented in ARLEQUIN (Schneider *et al.* 2000). For the same loci the possible presence of null alleles is suggested by MICRO-CHECKER (Van Oosterhout *et al.* 2004). However, this evidence is consistent across populations only for Test 10.

Locus Test 21 showed different allele size distributions in the two subspecies: the larger alleles observed in *T. h. boettgeri* and *T. h. hermanni*, were 235 pb and 307 pb, respectively. This pattern was confirmed also by analysing individuals of different populations (data not reported). Given the large range of size observed at this locus, the alleles 203 and 307, simultaneously found in one individual, were cloned and sequenced to exclude the presence of nonspecific band or insertion event. The flanking regions resulted identical and the size gap was entirely ascribable to a difference in the microsatellite repetition numbers.

The microsatellite loci here reported are the first ones identified directly in the genome of *T. hermanni* and may represent a relevant tool for the genetic investigation on this species.

Table 1 Characteristics of microsatellites loci in *Testudo hermanni* and variability in two populations of the two subspecies *Testudo hermanni hermanni* and *Testudo hermanni boettgeri*. For the two subspecies, variability was tested on 29 and 44 individuals, respectively. For each labelled primer the Dye is reported to a footnote within brackets.* Significant deviation from HWE ($P < 0.05$)

Locus	Primer sequences (5'–3')	Repeat motif	T_a	GenBank Accession no.	<i>Testudo hermanni hermanni</i>			<i>Testudo hermanni boettgeri</i>		
					No. alleles	Size range (bp)	H_O/H_E	No. alleles	Size range (bp)	H_O/H_E
Test 10	F-(TET)AGACTCTCTGTGATGGTAATAGCA	$(AC)_{15}(TA)_2(GA)_2$	60 °C	AY822052	4	194–228	0.069/0.102*	10	186–228	0.545/0.745*
	R-GATTTTCATFTGGCATATAAGACACA									
Test 21	F-AAACTGGCTGAAACCCAGC	$(CA)_{10}(CT)_5$	56 °C	AY822048	5	203–307	0.034/0.370*	9	203–235	0.705/0.777
	R-(HEX)TTGGGAGTTTGACTGATCTAGGA									
Test 56	F-(FAM)GATATGCAGGCAACAGGCT	$(CT)_6GCT(CA)_{12}$	56 °C	AY822049	2	203–205	0.345/0.552	3	199–205	0.523/0.502*
	R-CAGGAATCTGTGCATGATTGA									
Test 71	F-GATTTGGTCAATATAGAGGAGG	$(AC)_9$	56 °C	AY822050	2	126–130	0.034/0.068	3	126–130	0.409/0.505
	R-TGTTGTACTTACCTGTTCTGATCTATT									
Test 76	F-GAATCTAACTTTTCTCTGTGGAGC	$(CA)_8$	58 °C	AY822051	2	116–118	0.445/0.525	2	116–118	0.023/0.045
	R-(HEX)TCTTATTCATATCTGATCACAAGA									
Test 88	F-(HEX)TTTCCACAGAAAGGAGGC	$(TC)_{10}(AC)_8$	58 °C	AY822053	2	183–209	0.069/0.068	5	181–209	0.727/0.688
	R-CAAATTTGAATAACACAGATTTTCCC									

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References

- Bour R (1997) *Testudo hermanni*. In: *Atlas of Amphibians and Reptiles in Europe* (eds Gasc JP, Cabela A, Crnobrnja-Isailovic J *et al.*), Societas Europaea Herpetologica & Museum National d'Histoire Naturelle (IEGB/SPN), Paris.
- Guo S, Thompson E (1992) Performing the exact test of Hardy-Weinberg proportion for multiple alleles distribution. *Human Biology*, **66**, 591–600.
- Schneider S, Roessli D, Excoffier L (2000) *ARLEQUIN, version 2.000: A Software for Population Genetics Data Analysis*. Genetics and Biometry Laboratory, University of Geneva, Geneva, Switzerland.
- Van Oosterhout C, Hutchinson WF, Wills DMP (2004) MICRO-CHECKER: software for identifying and correcting genotyping errors in microsatellites data. *Molecular Ecology Notes*, **4**, 535–538.
- Vos P, Hogers R, Bleeker M *et al.* (1995) AFLP: a new technique for DNA fingerprinting. *Nucleic Acids Research*, **23**, 4407–4414.
- Zane L, Bargelloni L, Patarnello T (2002a) Strategies for microsatellite isolation: a review. *Molecular Ecology*, **11**, 1–16.
- Zane L, Patarnello T, Ludwig A, Fontana F, Congiu L (2002b) Isolation and characterization of microsatellites in the Adriatic sturgeon *Acipenser naccarii*. *Molecular Ecology Notes*, **2**, 586–589.