Cross-amplification of microsatellite loci for species of the genus *Testudo*

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European tortoises of the genus *Testudo* are becoming seriously threatened mainly due to habitat urbanization and illicit pet trade. In this study we tested the cross-amplifications of 23 microsatellite genetic markers (isolated from Testudinidae and Emydidae) in five (sub)species of the genus *Testudo*. A subset of 8–10 polymorphic loci was defined across the tested taxa, providing new tools for hybrid assignment, population genetic studies and parental tests.

Key words: cross-amplification, microsatellite, parentage analysis, population genetics, *Testudo* tortoises.

European tortoises of the genus *Testudo* are becoming endangered in the wild due to reduction and fragmentation of their habitat. Exploitation for the pet trade is another relevant factor that may lead to depletion of natural populations. As a consequence, several *Testudo* species were included in Annex II of the Habitats Directive (92/43/EEC) and the entire genus was placed on Appendix II of CITES (the Convention on International Trade in Endangered Species of Wild Fauna and Flora) for regulating its international trade. The European normative Reg. (EC) No. 865/2006 establishes that individuals belonging to CITES species can be traded only if they and their parents were born and bred in captivity. According to Article 55, breeders of CITES species are expected to prove, by genetic analysis, that traded individuals belong to the second generation (*F2*) of captive bred animals.

Here we tested 23 microsatellite loci for cross-amplification in four *Testudo* species: *T. hermanni*, subspecies *T. h. hermanni* (Gmelin, 1789) and *T. h. boettgeri* (Mojsisovics, 1889); *T. hercegovinensis* (Werner, 1899; *sensu* Perälä et al., 2002; Perälä et al., 2004; Bour, 2004); *T. graeca* (Linnaeus, 1758); and *T. marginata* (Schoepff, 1792). Of these 23 loci, fourteen were selected from Testudinidae species: Gal263, Gal50, Gal73, Gal75, Gal136 and Gal127 were described for *Geochelone* spp. (Ciofi et al., 2002); Ah01 and Ah02 for *T. horsfieldii* (Johnston et al., 2006); Leo10, Leo56, Leo76, Leo71, Leo21 and Leo88 for *T. hermanni* (Forlani et al., 2005). Since the set of six microsatellites isolated by Forlani et al. (2005) has already been tested on *T. h. hermanni* and *T. h. boettgeri,* these loci were tested for cross-amplification only on *T. hercegovinensis*, *T. marginata* and *T. graeca*; otherwise, a screening of polymorphism was performed on *T. hermanni* spp. Nine of the 23 loci selected (GmuB08, GmuD51, GmuD121, GmuD114, GmuD55, GmuD28, GmuA19, GmuD88 and GmuD87) were isolated for an Emydidae species (*Glyptemys muhlenbergii*: King & Julian 2004); three were already successfully used on *T. horsfieldii* (Johnston et al., 2006) and two on *T. graeca* (Roques et al., 2004).

Genomic DNA was extracted from epithelial cells by combining alkaline and temperature lysis. Samples were collected using buccal swabs, a non-invasive procedure that can be also employed in small-sized and young individuals (such as hatchlings) and that represents a valid alternative to invasive techniques such as blood sampling (Wingfield, 1999; Poschadel & Møller, 2004; Broquet et al., 2007).

We initially tested the potential of cross-amplification for all 23 loci with 3–5 samples taken from each species and subspecies. The PCR amplifications were performed in a Perkin Elmer 9600 thermal cycler, with the following cycle: 10 min of initial denaturation at 94° C followed by 35–40 cycles of denaturation at 94° C for 30 s, 45 s of annealing at 48–58° C, 1 min of elongation at 72° C and 30 min at 72° C for final extension. PCR amplifications were performed in 15 µl of final volume containing; over 2 µl DNA: 1.5 µl of buffer 10X (Invitrogen), 1.5–2 mM of $MgCl_2$, 0.3–0.5 µl of primers 10 µM, 200 µM of each dNTP, 0.3 units of Taq (Invitrogen). Amplification of each locus for each species was performed at different annealing temperatures and magnesium and primer concentrations in order to find the optimal PCR conditions for allele detection. One of the two primers of each pair was fluorescently labelled (FAM, HEX, NED) for detection on ABI 310 Applied Biosystems (sizing). PCR amplification was initially assessed on 1.5% agarose gel. Both detectable and undetected amplicons were resolved on automatic sequencer.

Out of the 23 microsatellite loci tested, 12 were discarded due to the absence of allele detection or to stutter products during their amplification, which cause unreliable individual genotyping. The remaining 11 gave repeatable and reliable genotyping results in all tested species/subspecies. PCR conditions are reported in Table 1 together with the original primer sequences, not modified for our cross-amplification tests.

Before performing the screening of polymorphism, these 11 microsatellite loci were sequenced for verifying the presence of the expected repeat motif. Sequencing was performed with the ABI BigDye terminator mix in an ABI Prism 310 Genetic Analyzer (Applied Biosystems), using as a template the DNA of a homozygote individual for each *Testudo* species/subspecies tested. The presence of the expected repeat motif was confirmed for all 11 loci in each species/subspecies, with the exception of locus GmuD87 in *T. marginata*, where we found the repeat motif $(TC)_n(ATCT)_n$ instead of the expected $(ATCT)_n$ (see King & Julian, 2004).

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Table 1. Primer sequences and optimal PCR conditions of 11 microsatellite loci successfully amplified in all *Testudo* species/subspecies tested. Ta=annealing temperature.

Locus	Primer sequences (5'–3')	Ta	$[Mg^{2+}]$	[primers]	N cycles
Leo10	$F_{(ned)}$-AGACTCTCTGTGATGGTAATAGCA R-GATTTTCATTGGCATATAAGACACA	50°C	2 mM	0.2 mM	40
Leo56	$F_{(fam)}$-GATATGCAGGCAAACAGGCT R-CAGGAATCTGTGCATGATTGA	54°C	2 mM	0.2 mM	40
Leo76	F-GAATTCTAACTTTTCTCTGTGGAGC $R_{(ned)}$-TCTTATTGCATATCTGAGTACAGAAGA	54°C	2 mM	0.2 mM	40
Leo71	F-GATTGTGGTCACATATAGAGGAGG $R_{(fam)}$-TGTTGTACTTAGCTGTTCTGATCTATT	55°C	2 mM	0.2 mM	40
Leo21	$F_{(fam)}$-AAACTGGCTGAAACCCAGC R-TTGGGAGTTTGACTGATCTAGGA	54°C	2 mM	0.2 mM	40
GmuB08	$F_{(hex)}$-CTCTGAGACCCTTATTCACGTC R-AGCCTTTGTCTGTAAGCTGTTC	55°C	2 mM	0.2 mM	40
GmuD51	$F_{(hex)}$-GTTGGGCACTAGATAGATTCG R-CATTCAAGTCAAGGGAAAGAC	55°C	2 mM	0.2 mM	40
GmuD121	$F_{(hex)}$-GGCAAATATCCAATAGAAATCC R-CAACTTCCTCGTGGGTTCAG	50°C	2 mM	0.2 mM	40
GmuD55	$F_{(hex)}$-GTGATACTCTGCAACCCATCC R-TTGCATTCAGAATATCCATCAG	50°C	2 mM	0.2 mM	40
GmuA19	$F_{(fam)}$-TAAGAGACAGATGCTCAGCAAG R-GTACATAACACGCACCCAATG	54°C	2 mM	0.2 mM	40
GmuD87	$F_{(fam)}$-AAACCCTAAGACATCAGACAGG R-CAAATCCAGTACCCAGAAAGTC	52°C	2 mM	0.2 mM	40

A total of 77 *Testudo* individuals were genotyped for further analyses of polymorphism, Hardy-Weinberg Equilibrium (HWE) and linkage disequilibrium. The number of alleles was calculated using FSTAT ver. 2.9.3 (Goudet, 1995). Linkage equilibrium among loci for each species/subspecies was assessed using GENEPOP ver. 3.4 (Raymond & Rousset, 1995), calculating *P*-values using a Markov chain with 1000 batches and 1000 iterations per batch, and applying the Bonferroni correction for multiple comparisons (significant corrected *P*-value=0.0009). GENEPOP was also used to calculate observed (*Ho*) and expected (*He*) heterozygosity. Deviations from HWE for each locus across each species/subspecies were assessed using a Markov chain with 1000 batches and 1000 iterations per batch. MICROCHECKER 2.2.3 (van Oosterhout et al., 2004) was used for detecting the existence of null alleles for each locus in all tested species/subspecies.

No linkage associations were evident from pairwise comparisons of loci with the exception of pairs Leo10/Leo71 for *T. graeca*. The results on polymorphism and HWE are reported in Table 2. GmuD55 was the only monomorphic locus in all species and subspecies. All other loci were polymorphic in at least 3 species/subspecies with a number of alleles ranging from 2 to 19 (Table 2). We found a slightly higher number of alleles for loci Leo10, Leo71, Leo56, Leo76 and Leo21 in *T. h. hermanni*, with respect to what was reported in Forlani et al. (2005), with a corresponding increase of size range. Conversely, for these loci we recorded a lower number of alleles with respect to Forlani et al. (2005) in *T. h. boettgeri*: this should be due to the lower number of individuals genotyped.

Three out of 10 polymorphic loci (Leo71, GmuB08 and GmuD121; Table 2) were at HWE in all species/subspecies. Regarding specific species/subspecies, we found sporadic cases of significant deviation from HWE for different loci, generally with an excess of homozygotes, (Table 2). The highest number of HWE deviations (4 loci out of 10: Table 2) was recorded for *T. h. hermanni* and *T. hercegovinensis*. These deficiencies of heterozygosity could be attributed to a non-panmictic population and/or to the presence of null alleles. Analysis by MICROCHECKER claimed the presence of scoring errors due to stuttering or large allele dropout for locus GmuD87 in *T. h. hermanni* and *T. marginata*, for locus Leo21 in *T. h. hermanni* and for loci Leo76 and Leo56 in *T. hercegovinensis*. These results suggested caution when using these loci for the species reported above.

Overall, this study showed that a total of 8–10 polymorphic loci per species exhibited the standard characteristics for being used as co-dominant markers in population genetic studies and hybrid assignment. Moreover, 3–7 loci per species, for which we detected at least 3 alleles, appeared potentially suitable for parental genetic analysis as required for trade regulations. This study confirmed the validity of cross-amplification tests across phylogenetically distant taxa, such as those belonging to different families. The possibility of applying identical microsatellite loci to different species allows to avoid the expensive phase of their *ex-novo* isolation.

Table 2. Polymorphism analysis, (*n*=number of individuals tested, Na=number of alleles), expected (*He*) and observed (*Ho*) heterozygosity for each locus in each species/subspecies. *P*=Hardy-Weinberg probability test (**P*<0.05).

Locus	Species	Size range	*n*	Na	*He*	*Ho*	*P*
Leo10	*T. hermanni hermanni*	178–232	35	11	0.846	0.742	0.045*
	T. hermanni boettgeri	174–228	6	6	0.848	0.666	0.074
	T. hercegovinensis	178–212	10	3	0.589	0.40	0.019*
	T. marginata	194–234	10	9	0.868	0.90	0.41
	T. graeca	178–230	11	7	0.852	0.909	0.034*
Leo56	*T. hermanni hermanni*	197–215	39	7	0.696	0.692	0.003*
	T. hermanni boettgeri	199–203	6	2	0.166	0.166	1.00
	T. hercegovinensis	199–207	8	3	0.425	0.125	0.015*
	T. marginata	202–208	9	2	0.424	0.333	1.00
	T. graeca	232–284	11	7	0.848	0.818	0.842
Leo76	*T. hermanni hermanni*	116–118	37	2	0.293	0.351	0.565
	T. hermanni boettgeri	116	6	monomorphic	/	/	/
	T. hercegovinensis	114–116	10	2	0.336	0.00	0.009*
	T. marginata	114	10	monomorphic	/	/	/
	T. graeca	112–114	10	2	0.189	0.00	0.052
Leo71	*T. hermanni hermanni*	122–130	37	5	0.642	0.81	0.376
	T. hermanni boettgeri	126–130	6	3	0.621	0.666	1.00
	T. hercegovinensis	128	10	monomorphic	/	/	/
	T. marginata	129–139	10	3	0.468	0.40	1.00
	T. graeca	121–161	11	7	0.865	1.00	0.682
Leo21	*T. hermanni hermanni*	200–312	37	8	0.738	0.378	0.00*
	T. hermanni boettgeri	232–280	5	2	0.466	0.60	1.00
	T. hercegovinensis	212–230	9	3	0.307	0.111	0.058
	T. marginata	206–234	6	8	0.924	0.666	0.085
	T. graeca	202–214	11	3	0.627	0.636	0.817
GmuB08	*T. hermanni hermanni*	206–227	37	7	0.704	0.729	0.083
	T. hermanni boettgeri	206–221	6	5	0.742	0.666	0.517
	T. hercegovinensis	206–245	10	6	0.731	0.60	0.106
	T. marginata	203–215	10	3	0.573	0.40	0.346
	T. graeca	221–245	11	4	0.671	0.909	0.39
GmuD51	*T. hermanni hermanni*	129–165	39	6	0.686	0.717	0.504
	T. hermanni boettgeri	137–165	6	4	0.636	0.666	0.756
	T. hercegovinensis	139–211	10	9	0.889	0.90	0.28
	T. marginata	130–146	10	4	0.50	0.30	0.028*
	T. graeca	138–226	11	8	0.87	1.00	0.923
GmuD121	*T. hermanni hermanni*	121	39	monomorphic	/	/	/
	T. hermanni boettgeri	121	6	monomorphic	/	/	/
	T. hergecovinensis	121–129	9	2	0.209	0.00	0.058
	T. marginata	125–133	10	3	0.636	0.40	0.135
	T. graeca	137–255	10	9	0.894	1.00	0.936
GmuD55	*T. hermanni hermanni*	138	37	monomorphic	/	/	/
	T. hermanni boettgeri	138	6	monomorphic	/	/	/
	T. hercegovinensis	138	10	monomorphic	/	/	/
	T. marginata	138	10	monomorphic	/	/	/
	T. graeca	138	11	monomorphic	/	/	/
GmuA19	*T. hermanni hermanni*	117–119	35	2	0.109	0.114	1.00
	T. hermanni boettgeri	117–121	6	3	0.53	0.166	0.03*
	T. hercegovinensis	119–135	10	4	0.71	0.90	0.026*
	T. marginata	121	9	monomorphic	/	/	/
	T. graeca	119–121	11	2	0.173	0.181	1.00
GmuD87	*T. hermanni hermanni*	210–294	35	19	0.916	0.742	0.01*
	T. hermanni boettgeri	210	5	monomorphic	/	/	/
	T. hercegovinensis	206–246	10	6	0.842	0.90	0.957
	T. marginata	300–472	10	12	0.926	0.60	0.001*
	T. graeca	268–342	10	10	0.91	0.70	0.031*

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