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Cross-amplification of microsatellite loci for the Mediterranean stripe-necked terrapin (*Mauremys leprosa*)

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Abstract. To accurately infer population structuring and manage species, it is advised to combine data obtained from mitochondrial DNA (mtDNA) with data from fast evolving markers such as microsatellites. To date, the evolutionary history of a threatened Mediterranean species, *Mauremys leprosa*, was inferred based solely on mtDNA data, which may lead to an incomplete, or partially explained, population structuring. We tested the cross-amplification of 16 microsatellite loci in 190 individuals of *M. leprosa* from six Iberian and two African populations. We obtained a successful set of 11 polymorphic loci with 2-18 alleles and observed heterozygosity ranging from 0.007-0.783. This panel of loci can be used for future research in *M. leprosa*, such as population structuring, analysis of gene flow in secondary contact zones, paternity analyses, changes in phenotypic traits and to assemble a comprehensive genetic dataset (mtDNA and nuDNA) that will allow the geographic assignment of individuals of unknown origin. These tools will help managing *M. leprosa* populations throughout the species' range.

Keywords: conservation genetics, cross-amplification, *Mauremys leprosa*, microsatellites, phylogeography, population genetics.

Inferring genetic structuring patterns based on molecular analysis of mitochondrial DNA usually produces an incomplete picture of the species' biogeographic processes due to its maternal heritability. These patterns are even more incomplete when studying species, such as turtles, with lower mtDNA mutation rate than other vertebrates, which is partially explained by their long life-span and average long generation time (Avise et al., 1992; Bromham, 2002; Lourenço et al., 2013). Therefore, the development and optimization of fast evolving nuclear markers such as microsatellite loci is crucial to unveil genetic diversity and structure patterns in this taxonomic group.

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The range of the Mediterranean stripe-necked terrapin *Mauremys leprosa* (Schweigger, 1812) embraces the Northwestern Africa and the Iberian Peninsula, with a few populations located in southwestern France (Keller and Busack, 2001). This species is currently threatened by habitat fragmentation and/or destruction, pet trade, alien species (Polo-Cavia, López and Martín, 2011) and pathogens (Hidalgo-Vila et al., 2008; Verneau et al., 2011). *Mauremys leprosa* is considered vulnerable by the IUCN and is listed in Appendix II of the Berne Convention and in Appendix II and IV of Habitat Directive (92/43/CEE) (Cox and Temple, 2009). A phylogeographic study of *M. leprosa* identified two major mitochondrial lineages (Fritz et al., 2006) classified in two subspecies: *M. l. saharica* (southern Morocco, eastern Algeria and Tunisia) and *M. l. leprosa* (Iberian Peninsula and northern Morocco). However, genetic differentiation was inferred solely from mtDNA data and population structuring within the two subspecies was not well resolved by the use of this marker. For developing conservation strategies and identification of management units, genetic population structure and diversity needs

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to be analysed in more detail. In this study, we tested and optimized a set of 16 microsatellite markers for *M. leprosa* that were developed for two closely related species (*M. caspica* and *M. rivulata*).

We tested 13 microsatellite loci developed for *M. caspica* (Vamberger, Stuckas and Fritz, 2011) and three microsatellite loci developed for *M. rivulata* (Mantziou et al., 2005) for cross-amplification in 190 *Mauremys leprosa* belonging to six Iberian (Algarve, Castro Verde, Castelo Branco, Caldas da Rainha, Madrid, Murcia) and two African populations (Ceuta and Tazia). Genomic DNA was extracted from blood using EasySpin (for *M. leprosa*) or Quiagen (for *M. caspica* and *M. rivulata*) extraction kits, following the manufacturer's protocol. In order to ensure that primers were amplifying correctly in *M. leprosa*, we used samples from *M. caspica* and *M. rivulata* as a positive control. We divided the PCR reaction in two multiplexes (see table 1); forward primers were labeled with fluorescent dye markers (FAM, NED, VIC and PET; Oetting et al., 1995). PCR amplifications were performed on 10 μl final volume containing 2 μl of DNA, 5 μl of Quiagen Multiplex PCR Kit and 0.14 μl-0.32 μl at 10 μM of each primer (table 1). Touchdown PCR conditions started with an initial denaturation for 95°C for 15 min (minutes) followed by 7 cycles of 95°C for 30 s (seconds), 58°C for 1 min (decreasing 0.5°C per cycle to 55°C), 72°C for 30 s; 24 cycles of 95°C for 30 s, 55°C for 1 min, 72°C for 30 s; 8 cycles of 95°C for 30 s, 53°C for 1 min, 72°C for 30 s, and a final elongation step at 60°C for 30 min. PCRs were performed on BioRad C1000 Thermocycler and genotyped on an ABI 3130*xl* genetic analyzer (Applied Biosystems, Foster City, CA, USA). GeneScan™-500 Liz was used as fragment size standard to score amplicons sizes on GeneMapper v4.0 (Applied Biosystems). GENEPOP v4.2 (Rousset, 2008) was used to assess deviations from Hardy-Weinberg equilibrium (H-WE) for each locus and population and linkage disequilibrium (LD) between loci at each population using the Markov chain method with 1000 batches and 10 000 iterations per batch. Bonferroni correction for multiple comparisons was applied for both cases. Observed and expected heterozygosities were calculated using GenAlEx v6.4 (Peakall and Smouse, 2006) and the possible existence of null alleles at each locus for all populations was assessed with MICROCHECKER v2.2.3 (Van Oosterhout et al., 2004).

All loci were in H-WE in all populations except for three cases that showed a deviation caused by heterozygote deficit (MC18 and MR-1 in Murcia, and MC20 in Tazia). Nevertheless, this pattern was not observed across all populations and therefore the loci were not discarded. The fact that we observed this pattern in only

Table 1. Characteristics of 16 microsatellite loci tested in *M. leprosa.* Loci whose codes begin with MR were designed for *Mauremys rivulata* and the ones that begin with MC were designed for *Mauremys caspica*. GenBank accession numbers are in brackets below each locus name abbreviation. Microsatellite repeat motif; Ta (C°) = PCR annealing temperature; Primer (μl) = quantity of primer (μl) of a 10 μM Primer solution; N = number of sampled individuals; Na = number of alleles; H_O = observed heterozygosity; H_E = expected heterozygosity; *P*-value (H-WE) = Hardy-Weinberg probability test (Fisher's exact test).

Multiplex	Locus	Repeat motif	Ta	Primer (μl)	N	Na	Allele size range (bp)**	H_O	H_E	*P*-value (H-WE)***
1	Mr-1 [AY934859]	$(AC)_{11}$	56	0.20	117	4	205-213	0.019	0.124	high sig.
1	* MC5 [HQ010418]	$(ATCT)_{12}$	56	0.32	156	15	184-244	0.773	0.805	0.6213
1	* MC6 [HQ010407]	$(ATCT)_{21}$	56	0.20	186	12	114-186	0.770	0.764	0.0063
1	MC8 [HQ010411]	$(AC)_{15}$	56	0.12	186	1	192	0	0	–
1	* MC12 [HQ010410]	$(TG)_{14}$	56	0.14	187	3	84-92	0.166	0.154	1
1	* MC17 [HQ010417]	$(TAGA)_8$	56	0.12	189	2	106-114	0.007	0.006	–
1	* MC22 [HQ010413]	$(CT)_6 \ldots (ATCT)_8$	56	0.14	189	6	100-112	0.562	0.542	0.2687
1	* MC24 [HQ010412]	$(AGAT)_8$	56	0.24	176	13	104-152	0.767	0.794	0.4777
2	MR-3 [AY934861]	$(GT)_8$	56	0.20	47	1	182	0	0	–
2	* MR-9 [AY934864]	$(CT)^{16}$	55.5	0.14	186	13	93-123	0.611	0.615	0.0185
2	* MC1 [HQ010420]	$(AGAT)_{12}$	56	0.14	189	2	89-105	0.02	0.018	–
2	* MC3 [HQ010419]	$(TAGA)_{14}$	56	0.24	175	18	195-263	0.783	0.8	0.2282
2	* MC18 [HQ010416]	$(ATCT)_{10}$	56	0.16	157	17	219-287	0.627	0.769	0.0158
2	* MC20 [HQ010415]	$(TATC)_{14}$	56	0.20	184	13	160-212	0.483	0.547	0.0099
2	MC21 [HQ010414]	$(TC)_{12}$	56	0.20	189	6	111-125	0.467	0.486	0.1821
2	MC25 [HQ010409]	$(AG)_{18}$	56	0.16	123	1	168	0	0	–

* Informative polymorphic microsatellite markers in *Mauremys leprosa*.

** We have corrected the allele sizes by discounting the fluorescent tail size to the allele size.

*** '–' corresponds to cases where the marker showed to be monomorphic or with only two alleles.

two populations might suggest that they could be inbred or that they are non-panmictic populations. Another explanation might be the presence of null alleles in MR1. This marker, together with MC21 showed signs of null alleles, since they have a low number of alleles sampled (MR1 – 4 alleles and MC21 – 6 alleles) and appeared as homozygotes in the majority of populations. No cases of linkage disequilibrium were found. None of the tested primers failed to amplify. However, three loci turned out to be monomorphic (MR-3, MC8 and MC25). Allele variation ranged from 2 (MC1, MC17) to 18 (MC3) (table 1). Overall, we successfully optimized for cross-amplification 11 polymorphic microsatellites in *M. leprosa*, which allow estimating fine scale genetic diversity and structuring across the species' distribution. In addition, past demographic events can also be assessed with these markers, allowing to draw a more detailed picture on the biogeographic history of the species. *Mauremys leprosa* shares its range with another freshwater terrapin, the European pond turtle (*Emys orbicularis*), across the Iberian Peninsula and northwestern Africa. The two species inhabit similar water bodies, although the latter is thought to be more sensitive against pollution. While the evolutionary history and contemporary genetic structure is well-studied in Iberian populations of *E. orbicularis* using evidence from mtDNA and microsatellites (Velo-Antón, García-París and Cordero Rivera, 2008), fast-evolving biparentally inherited microsatellites have never been used before for *M. leprosa*, which would be promising for a comparative phylogeographic study of both species. Moreover, the use of microsatellites and a comprehensive sampling would be particularly important for conservation studies because turtles are amongst the most common vertebrates associated to pet trade and illegal translocations (Van Dijk, Stuart and Rhodin, 2000; Moll and Moll, 2004), which has an important effect on specie's genetic structure and genetic variability within populations (Gong et al., 2009; Velo-Antón et al., 2011a). For instance, genetic characterization of Iberian populations of *E. orbicularis* allowed the allocation of unknown samples from Recovery Centres to the most likely region of origin (Velo-Antón et al., 2007) and we expect that this will be possible for *M. leprosa* as well using the cross-amplified microsatellite markers. For *M. leprosa*, many individuals are thought to be translocated to areas distant from their home populations across its entire distribution range, including terrapins from Morocco that were introduced to Iberian populations. Therefore, an accurate genetic characterization of *M. leprosa* populations will allow building a feasible tool to assign individuals of unknown origin to their natural populations, and help to better manage this species. Furthermore, we expect that these microsatellite markers will contribute to a better understanding of potential gene flow in the contact zone of the two subspecies in North Africa (e.g. Pedall et al., 2011 for *E. orbicularis*), paternity analyses (e.g. Roques et al., 2006) and changes in phenotypic traits (e.g. Velo-Antón, Becker and Cordero-Rivera, 2011b).

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References

Avise, J.C., Bowen, B.W., Lamb, T., Meylan, A.B., Bermingham, E. (1992): Mitochondrial DNA evolution at a turtle's pace: evidence for low genetic variability and reduced microevolutionary rate in the Testudines. Mol. Biol. Evol. **9**: 457-473.

Bromham, L. (2002): Molecular clocks in reptiles: life history influences rate of molecular evolution. Mol. Biol. Evol. **19**: 302-309.

Cox, N., Temple, H. (2009): European Red List of Reptiles. Office for Official Publications of the European Communities, Luxembourg.

Fritz, U., Barata, M., Busack, S.D., Fritzsch, G., Castilho, R. (2006): Impact of mountain chains, sea straits and peripheral populations on genetic and taxonomic structure of a freshwater turtle, *Mauremys leprosa* (Reptilia, Testudines, Geoemydidae). Zool. Scr. **35**: 97-108.

Gong, S.-P., Chow, A.T., Fong, J.J., Shi, H.-T. (2009): The chelonian trade in the largest pet market in China: scale, scope and impact on turtle conservation. Oryx **43**: 213-216.

Hidalgo-Vila, J., Díaz-Paniagua, C., Pérez-Santigosa, N., De Frutos-Escobar, C., Herrero-Herrero, A. (2008): Salmonella in free-living exotic and native turtles and in pet exotic turtles from SW Spain. Res. Vet. Sci. **85**: 449-452.

Keller, C., Busack, S.D. (2001): *Mauremys leprosa* (Schweigger, 1812). In: Handbuch Der Reptilien Und Amphibien Europas Band3/IIIA Schildkröten (Testudines) I (Bataguridae, Testudinidae, Emydidae), p. 57-58. Fritz, U., Ed., Wielbelsheim, Germany.

Lourenço, J.M., Glémin, S., Chiari, Y., Galtier, N. (2013): The determinants of the molecular substitution process in turtles. J. Evolution. Biol. **26**: 38-50.

Mantziou, G., Antoniou, A., Poulakakis, N., Goulielmos, G., Tsigenopoulos, C.S., Pinou, T., Mylonas, M. (2005): Isolation and characterization of six polymorphic microsatellite markers in the freshwater turtle *Mauremys rivulata* (Testudines: Geoemydidae). Mol. Ecol. Notes **5**: 727-729.

Moll, D., Moll, E. (2004): The Ecology, Exploitation, and Conservation of River Turtles. Oxford University Press, New York.

Oetting, W., Lee, H., Flanders, D., Wiesnes, G., Sellers, T., King, R. (1995): Linkage analysis with multiplexed short tandem repeat polymorphisms using infrared fluorescence and M13 tailed primers. Genomics **30**: 450-458.

Peakall, R., Smouse, P.E. (2006): GENALEX 6: genetic analysis in Excel. Population genetic software for teaching and research. Mol. Ecol. Notes **6**: 288-295.

Pedall, I., Fritz, U., Stuckas, H., Valdeón, A., Wink, M. (2011): Gene flow across secondary contact zones of the *Emys orbicularis* complex in the Western Mediterranean and evidence for extinction and re-introduction of pond turtles on Corsica and Sardinia (Testudines: Emydidae). J. Zool. Syst. Evol. Res. **49**: 44-57.

Polo-Cavia, N., López, P., Martín, J. (2011): Aggressive interactions during feeding between native and invasive freshwater turtles. Biol. Invasions **13**: 1387-1396.

Roques, S., Díaz-Paniagua, C., Portheault, A., Pérez-Santigosa, N., Hidalgo-Vila, J. (2006): Sperm storage and low incidence of multiple paternity in the European pond turtle, *Emys orbicularis*: A secure but costly strategy? Biol. Conserv. **129**: 236-243.

Rousset, F. (2008): GENEPOP'007: a complete re-implementation of the genepop software for Windows and Linux. Mol. Ecol. Res. **8**: 103-106.

Vamberger, M., Stuckas, H., Fritz, U. (2011): Fifteen microsatellite markers for the stripe-necked terrapin *Mauremys caspica* (Testudines: Geoemydidae) and cross-amplification tests in *M. rivulata*. Conserv. Genet. Res. **3**: 87-89.

Van Dijk, P.P., Stuart, B., Rhodin, A.G.J. (2000): Asian Turtle Trade: Proceedings of a Workshop on Conservation and Trade of Freshwater Turtles and Tortoises in Asia, pp. 1-164. Chelonian Research Foundation, Lunenburg.

Van Oosterhout, C., Hutchinson, W.F., Wills, D.P.M., Shipley, P. (2004): MICRO-CHECKER: software for identifying and correcting genotyping errors in microsatellite data. Mol. Ecol. Notes **4**: 535-538.

Velo-Antón, G., Godinho, R., Ayres, C., Ferrand, N., Rivera, A.C. (2007): Assignment tests applied to relocate individuals of unknown origin in a threatened species, the European pond turtle (*Emys orbicularis*). Amphibia-Reptilia **28**: 475-484.

Velo-Antón, G., García-París, M., Cordero Rivera, A. (2008): Patterns of nuclear and mitochondrial DNA variation in Iberian populations of *Emys orbicularis* (Emydidae): conservation implications. Conserv. Genet. **9**: 1263-1274.

Velo-Antón, G., Wink, M., Schneeweiß, N., Fritz, U. (2011a): Native or not? Tracing the origin of wild-caught and captive freshwater turtles in a threatened and widely distributed species (*Emys orbicularis*). Conserv. Genet. **12**: 583-588.

Velo-Antón, G., Becker, C.G., Cordero-Rivera, A. (2011b): Turtle carapace anomalies: the roles of genetic diversity and environment. PloS ONE **6**(4): e18714.

Verneau, O., Palacios, C., Platt, T., Alday, M., Billard, E., Allienne, J.-F., Basso, C., Du Preez, L.H. (2011): Invasive species threat: parasite phylogenetics reveals patterns and processes of host-switching between non-native and native captive freshwater turtles. Parasitology **138**: 1778-1792.

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