ONLINE RESOURCES

Characterization of nine new microsatellite loci for the marbled newt, *Triturus marmoratus*

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Triturus marmoratus is distributed throughout central, western and southern France as well as in a large portion of northern Iberia. Despite being categorized as of least concern by the IUCN, it is protected by the Annex IV of the EU Habitats Directive and the Annex III of the Bern Convention (Arntzen *et al.* 2009). As most amphibians, *T. marmoratus* is particularly sensitive to habitat loss and landscape fragmentation (Cushman 2006). Maintaining connectivity is therefore critical to ensure genetic diversity and sustainable populations. A good knowledge of the population genetic structure is necessary. Microsatellites are among the most commonly used genetic markers in numerous field of research (DeFaveri *et al.* 2013) and can be used in landscape genetics to assess population structure and quantify gene flow. Thus it can provide useful information for applied conservation ecology (Emel and Storfer 2015).

Total genomic DNA from epithelial cells obtained from buccal swab and from tissue muscle of 15 marbled newts was isolated using the DNeasy Blood and Tissue kit (Qiagen, Valencia, USA) pooled in equal quantities, and 1 μg of the pooled DNA was used for the development of microsatellites libraries through 454 GS-FLX Titanium pyrosequencing of enriched DNA libraries as described in Malausa *et al.* (2011). Briefly, total DNA was mechanically fragmented, ligated to standard adapters, enriched for AG, AC, AAC, AAG, AGG, ACG, ACAT and ATCT repeated motifs, and amplified by PCR. The purified products were then sequenced on a GsFLX PTP (Roche, Basel, Switzerland) following the manufacturer's instructions. One hundred and seventy-three microsatellite sequences were identified. From these, all sequences with an important number of repeats (as they have better chances to be polymorphic) and a size greater than 100 bp were selected. These resulted in the selection of 87 microsatellite sequences. Primers were designed with the QDD software (Meglécz *et al.* 2010).

Genotyping was realized by the Gentyane INRA plateform (Clermont-Ferrand, France) with three populations of 30, 29 and 23 individuals from the Pays de la Loire region (north of Nantes, south of Nantes and east of La Roche-sur-Yon), France. PCR amplification was performed in 10 μL reaction volumes containing 5 μL of AmpliTaq Gold® 360 Master Mix (AB-Life Technologies, Carlsbad, USA), 0.25 μL of forward primer, 0.25 μL of 5′ fluorescent labelled reverse primer and 2.5 ng of genomic DNA. Reactions were performed in a Veriti 384-Well Thermal Cycler (Applied Biosystems) with following conditions: 90°C for 10 min, 7 cycles at 95°C, 62°C (−1°C/cycle) and 72°C 30 s each, 30 cycles at 95°C, 55°C and 72°C 30 s each, then eight cycles at 95°C, 56°C and 72°C 30 s each and 72°C for 5 min. PCR products were then multiplexed and diluted (3 μL of each PCR product in 190 μL of water) and 2 μL of the dilution were added to 5 μL of a mix of formamide and 500LIZ Size Standard ladder in the manufacturer's recommended proportions for sequencing with a 3730xl DNA Analyzer (Applied Biosystems). Based on polymorphism and quality, only 10 of 87 microsatellites were preselected. They were then tested for the presence of null allele and linkage disequilibrium using the software GENEPOP (Raymond and Rousset 1995) on 82 individuals from three different sampling sites. No significant linkage disequilibrium was noted after standard Bonferroni correction. Locus Tmar6 had a high frequency of null alleles, ranging from 0.856 to 0.983 and was therefore removed from further analysis. Number of alleles (*N*a), observed heterozygosity (H_o) and expected heterozygosity (H_e) were calculated using the software Genetix ver. 4.05 (Belkhir *et al.* 1996) and are reported in table 1, along with *P* values for tests of departure from Hardy–Weinberg equilibrium (HWE) (performed in GENEPOP). As for previous microsatellite markers developed for the congeneric species

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Table 1. Characterization of nine polymorphic microsatellite loci for the marbled newt (*T. marmoratus*) ($N = 82$).

Locus	Repeat motif	Primer sequence (5′–3′)	Primer dye	Size range (bp)	N_a	H_E	H_O
Tmar02	$(TAGA)_8$	F: CACTCTTCCCTTCAACAGGC R: CACTCTTCCCTTCAACAGGC	FAM	110–153	12	0.7001	0.5758 (0.0020)**
Tmar04	$(TC)_{10}$	F: AACAAGCTGGTAAGCCGGTA R: CCTGAGCTAGAATGGAAGACAAA	VIC	145–154	2	0.4239	0.3171 (0.9072)
Tmar09	$(CA)_{15}$	F: TTTCTGACAAGAAGCCTCCC R: AGACCTGACAGATGTAGCCCA	FAM	177–195	7	0.1632	0.1379 (0.0671)
Tmar17	$(AAG)_{20}$	F: TCCAACGTGCTTTCATTTCA R: GCACATTGGAAAGTGTTCCC	VIC	204–213	3	0.2335	0.1646 (0.1508)
Tmar20	$(AGAT)_{17}$	F: GTCTGTCTGTCATCCATCTATCC R: CAGACAGGCAGGCAGAGATA	NED	196–238	10	0.7986	0.8049 (0.4609)
Tmar21	$(AGAT)_{16}$	F: TGGTGTCATACGTTGTAGGCA R: TCAATGTAATCCAAGAGAAGGTCA	NED	268–287	6	0.6296	0.5385 (0.4676)
Tmar22	$(AGAT)_{15}$	F: GGAAAGATATGTGCTGGTCCC R: CGAGAGAGGATGGATGGATG	NED	151–163	4	0.5238	0.5062 (0.4471)
Tmar23	$(AC)_{15}$	F: CAAGGATCAACTATGCATCCAG R: AAATAATCTACCACAAAGAGACCATT	PET	207–299	6	0.0862	0.0633 (0.0206)*
Tmar27	$(AC)_{14}$	F: ATTTTCATGGAAAACATGCG R: GGCAAAGTGAGCAAGAGACC	PET	186–192	3	0.1589	0.1235 (0.0819)

N_a, number of alleles observed; H_O, observed heterozygosity; H_E, expected heterozygosity P values for the exact tests of departure from HW proportions (H1 heterozygosity deficit) are provided in parenthesis. *$P < 0.05$, **$P < 0.0056$ (Bonferroni correction).

T. cristatus (Krupa *et al.* 2002), a typical range of microsatellite polymorphism was found (2 to 12 alleles per locus; highly variable H_O, ranging from 0.0633 to 0.8049). After standard Bonferroni correction, only Tmar2 showed significant ($\alpha = 0.0056$) departure from HWE. The microsatellite loci published in this study are currently used to analyse the genetic structure of the marbled newt and levels of landscape fragmentation in different regions of western France.

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