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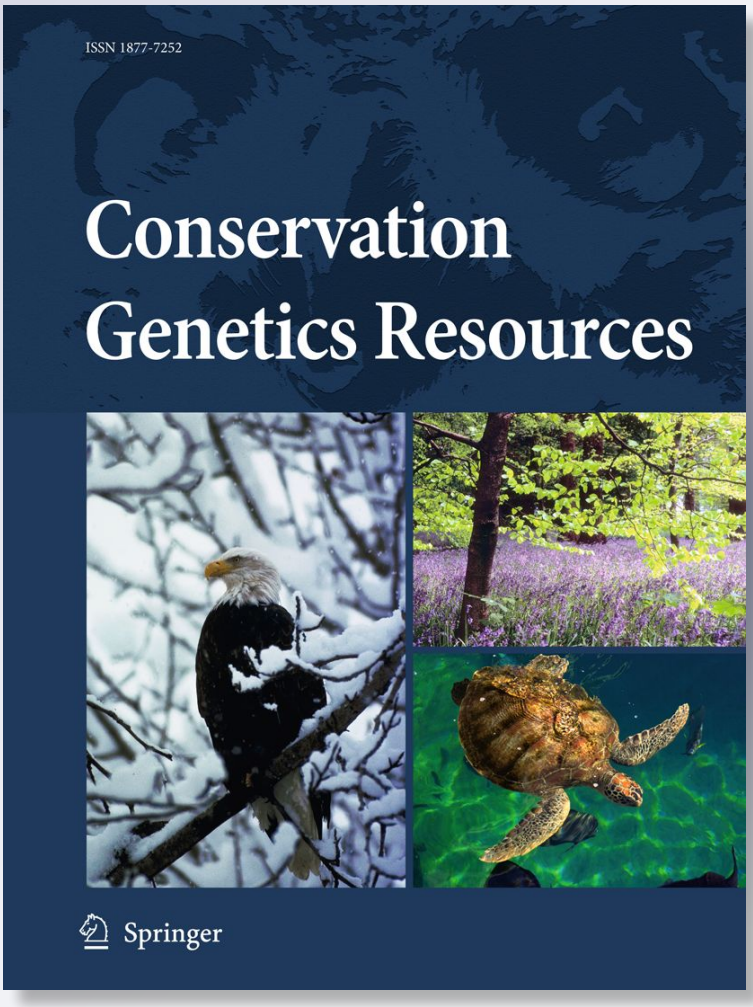

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TECHNICAL NOTE

Characterization of 13 microsatellite loci for the Pygmy Marbled Newt *Triturus pygmaeus* (Salamandridae)

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Abstract We have developed thirteen polymorphic microsatellite loci for the newt *Triturus pygmaeus* using an enriched-library approach. We have detected 171 alleles in 60 individuals genotyped (mean number of alleles per locus was 13.15) in two different populations in the Doñana National Park, Southwestern Spain. Expected heterozygosities ranged from 0.16 to 0.956 in one population (Mata de los Domínguez) and from 0.1 to 0.959 in the other population (Navazo de la Higuera). The levels of polymorphism of the developed markers render them readily applicable for population genetic studies of diversity, structure, and migration.

Keywords Triturus pygmaeus · Doñana National Park · Iberian Peninsula · SSR

Amphibians are one of the vertebrates groups most suited to test metapopulation and sink/source dynamics. This is mainly due to their life style linked to ponds, often temporary and surrounded by unfavorable habitat. In urodeles, for which no long-distance dispersal is assumed (Smith and Green 2005), the development of molecular markers to assess gene flow, especially in fragmented habitats, is particularly useful. The taxonomic status of Pygmy Marbled Newt, *Triturus pygmaeus*, (Wolterstorff 1905), was raised recently to species level, becoming separated from his sister species, *Triturus marmoratus* (García-París et al. 2001). The Pygmy Marbled Newt is an endemic species of the Iberian Peninsula, which inhabits the central and southern part of Portugal and the southwestern quarter of Spain (García-París 2002). This small newt occupies mainly freshwater marshlands within Mediterranean shrublands (García-París 2002). These marshland areas are also dedicated to agriculture, becoming increasingly affected by fragmentation and desiccation.

With the aim of studying gene flow and dispersal patterns in dynamic metapopulation settings in marshland areas, we undertook the search for polymorphic microsatellite loci in the Pygmy Newt *Triturus pygmaeus*.

We developed microsatellite libraries following Jones et al. (2002). We extracted approximately 100 μg of genomic DNA of one individual using the QIAGEN DNeasy Tissue Extraction kit. The DNA was partially restricted with seven blunt-end restriction enzymes (*Rsa*I, *Hae*III, *Bsr*B1, *Pvu*II, *Stu*I, *Sca*I and *Eco*RV). Fragments (300–750 bp) were ligated to 20-bp oligonucleotides containing a *Hin*dIII site at the 5′ end, and subjected to magnetic bead capture.

Four libraries were prepared in parallel using Biotin-CA_{15}, Biotin-GA_{15}, Biotin-ATG_{12} and Biotin-AAC_{12} as capture molecules (CPG Inc.). Captured molecules were amplified and restricted with *Hin*dIII to remove the adapters, and the resulting fragments were ligated into the *Hin*dIII site of pUC19 plasmid and introduced into *Escherichia coli* DH5α by electroporation. One hundred recombinant clones were selected at random for sequencing and 73 of them contained a microsatellite sequence. Polymerase chain reaction (PCR) primer pairs were designed for 44 clones using Designer PCR 1.03 (Research Genetics Inc.).

For marker evaluation, we isolated DNA from toe clips from 30 adult individuals collected from two populations: Navazo de la Higuera (NH) and Mata de los Domínguez (MD), both in the National Park of Doñana, Huelva

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Province, (Spain), using a standard phenol/chloroform extraction DNA protocol (Sambrook et al. 1989). Polymerase chain reactions were performed in final volume of 20 μL containing 1× buffer [67 mM Tris–HCl pH 8.8, 16 mM $(NH_4)_2SO_4$, 0.01% Tween-20], 2.5 mM $MgCl_2$, 0.01% BSA (Roche Diagnostics), 0.25 mM dNTPs, 0.40 μM dye-labelled M13 primer, 0.40 μM tailed-reverse primer, 0.04 μM M13 tailed-forward primer, 0.5 U *Taq* DNA polymerase (Bioline) and 50 ng of genomic DNA. Samples were amplified using a 'touchdown' PCR in a BIO-RAD DNA Engine Peltier Thermal Cycler, with an initial denaturation step of 2 min at 94°C; 17 cycles at 92°C for 30 s, annealing at 60–44°C for 30 s (1°C decrease in each cycle) and extension at 72°C for 30 s; 25 cycles at 92°C for 30 s, 44°C for 30 s and 72°C for 30 s with a final extension for 5 min at 72°C. Amplified fragments were analyzed on an ABI 3130xl Genetic Analyser and alleles were scored using GeneMapper 4.0 (Applied Biosystems) and LIZ 500 size standard.

We tested a total of 38 primer pairs from which we finally retained 13 polymorphic markers after inspecting their observed and expected heterozygosities (Cervus 3.0; Kalinowski et al. 2007) and testing for deviations from Hardy–Weinberg equilibrium, gametic disequilibrium (GenePop 4.0; Rousset 2007), and the presence of null alleles (Micro-Checker 2.2.3; Oosterhout et al. 2004). We used Bonferroni corrected *P* values to assess the significance of multiple tests.

Table 1 Primer sequences and repeat motif of the 13 polymorphic microsatellite loci isolated from the species *Triturus pygmaeus,* and polymorphism data in two populations of Doñana National Park: Navazo de la Higuera (NH) and Mata Domínguez (MD), in the Province of Huelva, Spain

Locus	Sequence (5′-3′)	Repeat structure	Size (bp)	MD (N = 30)				NH (N = 30)			
				K	Ho	He	HW	K	Ho	He	HW
TpygA8	F: TTCACATGCCTGAATCAGAAC R: CCGAAACTTTCAAGACAGGAG	$(AC)_{21}$	274–318	12	0.621	0.702	0.068	10	0.567	0.741	<0.001
TpygA12	F: ATAAATTGCCCAACCTCACTA R: GCACATGCACTTTAGAACAAG	$(GT)_{12}$	237–241	3	0.533	0.524	0.610	2	0.533	0.506	0.749
TpygA130	F: CCACCTGGAAAATGTCACC R: GCCTCACCTAAAGCCCACT	$(GA)_{14}AGAGG$ $(GA)_5$	178–200	5	0.567	0.614	0.337	4	0.667	0.691	0.009
TpygB2	F: GCACAAACCAACCCACTC R: AGGAGGAAGGTAGAAAGAAAGC	$(CT)_6TTCTGT(CT)_5$	167–171	2	0.172	0.16	1	2	0.2	0.183	1
TpygB9	F: ATCAAGAAACCTACAGCAACAC R: GCCTCGTCAAATACTGGAA	$(TC)_{16}$	188–195	3	0.136	0.21	0.138	2	0.103	0.1	1
TpygG104	F: TTCACACACTGAGCCCTTATG R: GGGGATGTATCTTCCTGTCC	$(TAGA)_{11}$	272–334	12	0.833	0.828	0.012	6	0.767	0.692	0.951
TpygG108	F: CGGAGTTTACAACCGATACC R: GAGTGGAAGAGGACGAACAG	$(ATCT)_{21}$	274–383	23	0.964	0.956	0.496	20	0.926	0.959	0.243
TpygG111	F: GTTGGCAATGCGACTTTG R: CATGAGGACACCACCAGAGA	$(TAGA)_{30}$	378–434	12	0.897	0.878	0.433	6	0.8	0.784	0.443
TpygG112	F: AGGAGGGACTGTTACTATGCC R: GAGCCCGTTTGTTTTTACAC	$(TATC)_{13}$	177–277	20	0.964	0.954	0.747	18	0.92	0.887	0.638
TpygG116	F: CAGTTCTATGAAGCCCATCAG R: CCCAAAAGATTGAGTGAAGTG	$(TAGA)_{11}$	210–238	12	0.828	0.86	0.149	8	0.833	0.809	0.816
TpygG134	F: ACATGCAACGTAGATGGATAC R: ACAAACTAAGCTCTGGTCTGAG	$(GATA)_{28}$	291–378	15	0.9	0.916	0.470	17	0.967	0.92	0.947
TpygG140	F: CCTCTGCTGAACCATTTTC R: TGGGCTCCAATATAAGAGC	$(CAGA)_{14}CTGA$ $(CAGA)_9$ $(TAGA)_{18}$	229–333	19	0.667	0.944	<0.001	10	0.667	0.88	0.002
TpygG141	F: AAGAGGTTTGGGATGAGTCAG R: GGCTCCCTTGAGTGTAATTTG	$(TATC)_{11}GATC$ $(TATC)_8$	270–320	12	0.833	0.868	0.4025	11	0.833	0.858	0.224

K number of alleles, *Ho* observed heterozygosity, *He* expected heterozygosity, *HW* nominal *P* values for the test of deviations from Hardy–Weinberg equilibrium. PCR products were indirectly labelled using FAM (Sigma–Aldrich), VIC, NED or PET (Applied Biosystems) dyes on an additional 19-bp M13 primer (5′-CACGACGTTGTAAAACGAC-3′) following Boutin-Ganache et al. (2001). A sequence tail (5′-GTGTCTT-3′) was added to the 5′ end of the reverse primer to improve adenylation and facilitate genotyping. Only stretches of more than four repeats are reported in the Repeat structure column. GenBank accession numbers are HQ 877828-HQ 877841

From the 13 loci selected, we detected a total of 150 alleles in MD and 116 alleles in NH, with individual locus values ranging from 2 to 23 and 2 to 20 alleles, respectively. The expected heterozygosity ranged from 0.16 to 0.956 in MD and from 0.1 to 0.959 in NH (Table 1). Two loci (TpygA8, TpygG140) in the population NH and one locus (TpygG140) in the population MD showed a significant deviation from Hardy–Weinberg equilibrium after Bonferroni correction ($P < 0.05/13 = 0.0038$). The presence of null alleles was suggested by Micro-Checker as a likely cause for the observed heterozygote deficit for the locus TpygG140 in both populations and for the locus TpygA8 in NH.

The microsatellite markers described in this study provide a valuable tool for the analyses of genetic diversity, structure and gene flow in the Pygmy Marbled Newt in the fragmented marshland areas where they live.

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