

VOLUME 4
NUMBER 4
DECEMBER
2004

MOLECULAR ECOLOGY

NOTES



Published by
Blackwell Publishing

PRIMER NOTE

Isolation and characterization of polymorphic tetranucleotide microsatellite loci in the Fire salamander *Salamandra salamandra* (Amphibia: Caudata)

SEBASTIAN STEINFARTZ,* DANIEL KÜSTERS and DIETHARD TAUTZ

*University of Cologne, Institute of Genetics, Weyertal 121, D-50931 Cologne, Germany***Abstract**

Ten tetranucleotide and one dinucleotide polymorphic microsatellite loci were cloned and characterized for the Fire salamander (*Salamandra salamandra*) from 34 populations in Germany. A high genetic diversity (5–22 alleles per locus) and heterozygosity (40.6–95.2%) were observed for these markers. Chord distances for population comparisons of the western evolutionary recolonization lineage in the area near Cologne ranged from 0.139 to 0.366, whereas population comparisons between the western and eastern lineage ranged from 0.541 to 0.670. When compared with classical isolation methods, a sufficient number of polymorphic microsatellites can be obtained for the Fire salamander only from specially enriched sublibraries.

Keywords: (GATA) motif enrichment, *Salamandra salamandra*, Salamandridae, tetranucleotide microsatellite loci

Received 19 February 2004; revision accepted 18 May 2004

The Fire salamander *Salamandra salamandra* represents an interesting and challenging species and subspecies complex with a long-standing interest in the evolution of their reproductive behaviour and correlated life history traits (Thiesmeier 1992; Dopazo & Alberch 1994). Moreover, it displays a high morphological and genetic diversity throughout its distribution range (Klewen 1991; Veith 1994; Steinfartz *et al.* 2000). *Salamandra salamandra* also seems to be a promising amphibian species to study the impact of environmentally enforced natural selection leading to divergent evolution of life history traits (Weitere *et al.* 2004). Finally, the phylogeography of *S. salamandra* offers interesting insights into the general recolonization patterns of vertebrates during and after Pleistocenic glaciations (Steinfartz *et al.* 2000; Hewitt 2001).

Initial attempts with standard protocols for cloning microsatellites (Tautz 1989; Rassmann *et al.* 1991) did not yield the expected fraction of positive clones and only one useful dinucleotide repeat locus was eventually obtained. Salamanders, like many amphibians, have heavily enlarged

genomes when compared to other vertebrates. We assume that this leads to a relative dilution of microsatellites by about 10-fold with respect to normal frequencies. Thus, we employed an enrichment protocol with a special focus on (GATA)_n tetranucleotide stretches, because these seem to occur frequently (Epplen *et al.* 1998) and because tetranucleotide repeats are generally easier to type.

Fresh tissue samples of *Salamandra salamandra terrestris* from the Kottenforst near Bonn (North-Rhine Westfalia, Germany) were used to extract total genomic DNA using the sodium dodecyl sulfate (SDS)–proteinase K/Phenol–Chloroform extraction method. Genomic DNA was stored in Tris-EDTA buffer (10 mM Tris-HCl, 0.1 mM EDTA, pH 8.0) and used for all subsequent reactions. The enrichment protocol followed essentially the FIASCO method (Zane *et al.* 2002) with the following modifications: (i) a 450–1000 bp fraction of the amplified fragment length polymorphism (AFLP) digestion–ligation reaction was cut from an agarose gel and this fraction was rerun and isolated again; (ii) PCR cycles to amplify *Mse*I adaptor flanking fragments were reduced to 20 cycles; (iii) 500 ng DNA from this enrichment step was mixed with 100 pmol of a 5′-biotinylated (GATA)₁₀ oligonucleotide probe in a total volume of 100 µL [4.2 × saline sodium citrate buffer (SSC), 0.07% SDS] at 67 °C for 2 h; (iv) 200 µL of Dynabeads

Correspondence: Sebastian Steinfartz. *Present address: Department of Ecology and Evolutionary Biology, Yale University, PO Box 208106, New Haven, Connecticut 06520–8160, USA. Fax: +1 203 432 6066; E-mail: Sebastian.Steinfartz@yale.edu

Table 1 Characterization of 11 Fire salamander (*Salamandra salamandra*) microsatellite loci. H_O , observed heterozygosity; H_E , expected heterozygosity; N , number of individuals typed

Locus	Primer sequences (5'–3'), fluorescence labelling	Repeat motif	Size range (bp)	No. of alleles	Annealing temp/ extension step	H_E	H_O	N
Sal E2 AY612894	F: EX-CACGACAAAATACAGAGAGTGGATA R: ATAT T TGAAAT TGCCCAT T TGGTA	(GATA) ₆ (GACA) ₅ (GATA) ₁₂	210–302	19	53.6 °C–1 min	0.872	0.952	1144
Sal E5 AY612884	F: CCACATGATGCCTACGTATGT TGTG R: FAM-CTCCTGT T TACGCT TCACCTGCTCC	(GT) ₁₄	182–194	5	63 °C–1 min	0.573	0.50	1187
Sal E6 AY612885	F: FAM-GGACTCATGGTCACCCAGAGGT TCT R: ATGGAT TGTGTCGAAATAAGGTATC	(GATA) ₂ GATG(GATA) ₁₅	284–304	6	59 °C–1 min	0.415	0.386	1174
Sal E7 AY612886	F: HEX-T T TCAGCACCAAGATACCTCT T T TG R: CTCCTCCATATCAAGGTCACAGAC	(GATA) ₆ (GACA) ₁₁ (GATA) (GACA)(GACA)(GATA) ₁₂	154–250	15	54.5 °C–1 min	0.917	0.982	1164
Sal E8 AY612887	F: FAM-GCAAAGTCCATGCT T TCCCT T TCTC R: GACATACCAAAGACTCCAGAATGGG	(TATC) ₁₆	138–214	15	59 °C–0.30 min 72 °C–0.30 min	0.870	0.790	1184
Sal E11 AY612888	F: FAM-CACAGT TCAT TAT T TCCACTACTGA R: AGGACCTCAAGACCTGGCTCT TCAA	(CTAT) ₁₅ CCAT(CTAT) ₅	224–280	10	59 °C–0.20 min 72 °C–0.30 min	0.563	0.537	1147
Sal E12 AY612889	F: TET-CTCAGGAACAGTGTGCCCAAAATAC R: CTCATAAT T TAGTCTACCTCCCAC	(CTAT) ₁₅	162–314	22	59 °C–0.20 min 72 °C–0.30 min	0.906	0.895	1143
Sal E14 AY612890	F: TET-GCTGCCCTCTCTGCCTACTGACCAT R: GCCAAGACATGGAACACCCCTCCCGC	(CTAT) ₁₆	150–262	12	69 °C–0.30 min 72 °C–0.40 min	0.565	0.473	1150
Sal 3 AY612891	F: FAM-CTCAGACAAGAAATCCTGCT TCT TC R: ATAAATCTGTCTGT TCCTAATCAG	(GAGT) ₁₅	182–258	16	61 °C–0.45 min	0.506	0.406	1100
Sal 23 AY612893	F: HEX-TCACTGT T TATCT T TGTTC T T T TAT R: AAT TAT T TGT T TGAGTCGAT T T TCT	(GACA) ₈ (GATA) ₄	280–320	11	50.4 °C–0.45 min	0.679	0.609	1082
Sal 29 AY612892	F: TET-CTCT T TGACTGAACCAGAACCCC R: GCCTGTCCGGCTCTGTGTAACC	(GATA) ₁₄	150–190	8	58 °C–0.45 min	0.803	0.946	1183

M-280 Streptavidin beads solution (DYNAL) were washed with $1 \times$ phosphate-buffered saline, 0.1% bovine serum antigen and subsequently resuspended at 67 °C in 400 μ L ($4.2 \times$ SSC, 0.07% SDS solution), then 200 μ L of bead solution was added to the fragment–probe mix and incubated for 30 min at 67 °C; (v) low stringency washes were performed four times with $1 \times$ SSC, 0.01% SDS at 38 °C. Each wash lasted 5 min. High stringency washes close to the dissociation equilibrium of the DNA fragment/(GATA)₁₀ complex were performed four times in $0.06 \times$ SSC, 0.01% SDS at 38 °C each for 10 min. The further steps were done according to the standard protocol. Enriched fragments were eventually ligated into pBluescript II vector (Stratagene) and transformed into electrocompetent SOLR-cells (Stratagene). Cells were plated on Luria-Bertani plates and nonradioactively screened with a biotinylated (GATA)₁₀ probe. Positive clones were sequenced with the T3 primer (5'-AATTAACCCTCACTAAAGGG-3') or T7 primer (5'-GTAAAACGACGGCCAGT-3').

Primer sequences flanking 11 *S. salamandra* microsatellites were designed using the OLIGO 4.0 software (National Biosciences, Plymouth, MN, USA). PCR reactions were performed in 20- μ L volumes: 100–250 ng of genomic DNA, $1 \times$ Eurobiotaq® PCR buffer, 1.5 mM MgCl₂, 0.5 mM dNTPs, 0.3 μ M of each primer (forward primer fluorescently labelled with FAM, HEX or TET), 1 U of Eurobiotaq® polymerase. The PCR profile for all loci was the same, except for the annealing and extension step (see Table 1): (i) 95 °C for 2 min; (ii) annealing temperature for extension step; (iii) 72 °C for 1 min; (iv) 95 °C for 0.30 min; (v) return to step (ii) for 33 times; (vi) 72 °C for 10 min. PCR products were run on a MegaBace 1000 sequencer (Amersham) and analysed with the Genetic Profiler software version 2.0.

Thirty-four *S. salamandra* populations were genotyped for 11 microsatellite loci. With the exception of one population from Bavaria representing the eastern recolonization lineage, all populations were derived from the western recolonization lineage (according to Weitere *et al.* 2004) and are located in an area near to Cologne (North Rhine Westfalia, Germany). Locus designation, repeat motif, primer sequences, size range of alleles, allelic diversity per locus, expected (H_E) and observed (H_O) heterozygosity are summarized in Table 1. Population differentiation, departure from Hardy–Weinberg equilibrium (HWE) and linkage disequilibrium were analysed using ARLEQUIN 2.00 (Schneider *et al.* 2000). Chord distances for population comparisons of the western evolutionary recolonization lineage in the area near Cologne ranged from 0.139 to 0.366, whereas population comparisons between the western and eastern lineage ranged from 0.541 to 0.670. We tested 34 populations for departure from HWE ($P = 0.05$; percentage given in brackets): Sal E2 (0.17), Sal E5 (0.2), Sal E6 (0.0), Sal E7 (0.13), Sal E8 (0.17), Sal E 11 (0.23), Sal E12 (0.13), Sal E14 (0.13), Sal 3 (0.0), Sal 23 (0.27), Sal 29 (0.1). All loci were

also tested for linkage disequilibrium for 34 populations ($P = 0.05$; percentage given in brackets): Sal E2 (0.24), Sal E5 (0.1), Sal E6 (0.18), Sal E7 (0.18), Sal E8 (0.14), Sal E11 (0.15), Sal E12 (0.18), Sal E14 (0.16), Sal 3 (0.13), Sal 23 (0.18), Sal 29 (0.12).

We conclude that the cloned microsatellite loci for the Fire salamander are a useful tool to detect genetic differentiation on the level of subpopulations and between populations. However, cloning of these loci did require an unusually high effort, which may be a typical problem for amphibian species. The parallel application of a very similar protocol for a fish species yielded 10–50 times more positive clones. Thus, although the application of microsatellites in amphibians will be equally useful as for other species, an extra effort for cloning has to be anticipated.

Acknowledgements

This project was funded by a grant of the Deutsche Forschungsgemeinschaft (DFG) to DT and SS (Ta99/16–1). We are thankful to Arne Nolte for helpful discussions and suggestions on the procedure of microsatellite cloning. Thanks also to Viktoria Rivkin and Kathrin Schwertz for their support.

References

- Dopazo H, Alberch P (1994) Preliminary results on optional viviparity and intrauterine siblicide in *Salamandra salamandra* populations from Northern Spain. *Mertensiella*, **4**, 125–137.
- Epplen JT, Maueler W, Santos EJ (1998) On GATAGATA and other 'junk' in the barren stretch of genomic desert. *Cytogenetics and Cell Genetics*, **80**, 75–82.
- Hewitt GM (2001) Speciation, hybrid zones and phylogeography – or seeing genes in space and time. *Molecular Ecology*, **10**, 537–549.
- Klewen R (1991) *Die Landsalamander Europas, Teil 1*. Die neue Brehm-Bücherei, Wittenberg Lutherstadt.
- Rassmann K, Schlötterer C, Tautz D (1991) Isolation of simple sequence loci for use in polymerase chain reaction-based DNA fingerprinting. *Electrophoresis*, **12**, 113–118.
- Schneider S, Roessli D, Excoffier L (2000) *ARLEQUIN: a Software for Population Genetics Data Analysis*, Version 2.000. Genetics and Biometry Laboratory, University of Geneva, Geneva, Switzerland.
- Steinfartz S, Veith M, Tautz D (2000) Mitochondrial sequence analysis of *Salamandra* taxa suggests old splits of major lineages and postglacial re-colonization of Central Europe from distinct source populations of *Salamandra salamandra*. *Molecular Ecology*, **9**, 397–410.
- Tautz D (1989) Hypervariability of simple sequences as a general source for polymorphic DNA markers. *Nucleic Acids Research*, **17**, 6463–6471.
- Thiesmeier B (1992) *Ökologie Des Feuersalamanders*. Westarp Verlag, Essen.
- Veith M (1994) Morphological, molecular and life history variation in *Salamandra salamandra*. *Mertensiella*, **4**, 355–397.
- Weitere M, Tautz D, Neumann D, Steinfartz S (2004) Adaptive divergence versus environmental plasticity: tracing local genetic adaptation of metamorphosis traits in salamanders. *Molecular Ecology*, **13**, 1665–1677 (in press).
- Zane L, Bargelloni L, Patarnello T (2002) Strategies for microsatellite isolation: a review. *Molecular Ecology*, **11**, 1–16.