# Short Notes

## Characterization of microsatellite loci in the Carpathian newt (*Lissotriton montandoni*)

**Krystyna Nadachowska, Ilona Flis & Wiesław Babik**

*Jagiellonian University, Institute of Environmental Sciences, Kraków, Poland*

Seven polymorphic microsatellite loci were developed for the Carpathian newt (*Lissotriton montandoni*) and tested for cross-amplification in multiple geographic groups of its sister species *L. vulgaris*. Genetic variation was characterized for 52 *L. montandoni* from two sites from Poland and Romania, reflecting the geographic range of the species. The number of alleles per locus ranged from six to 13 and the observed heterozygosity ranged from 0.20 to 0.87. Significant excess of homozygotes detected at two loci may suggest the presence of null alleles. No evidence for linkage disequilibrium between loci was detected. The cross-amplification success was variable, suggesting that the use of the markers developed in the present study may be limited to geographically restricted groups of the smooth newt.

*Key words*: cross-species amplification, microsatellites, Poland, Romania, smooth newt

The Carpathian newt (*Lissotriton montandoni*) is endemic to the Carpathians and easternmost Sudetes Mountains (Zavadil et al., 2003). The species exhibits little morphological variation and breeds mainly in ephemeral water bodies, e.g. in wheel ruts on mountain roads. In early spring the adults enter water bodies where courtship and mating take place and eggs are deposited. The Carpathian newt is the most terrestrial of all European newts (Zavadil et al., 2003). Despite significant morphological and ecological differences (Babik et al., 2003, 2005; Schmidtler & Frantzen, 2004; Nadachowska & Babik, 2009), *L. montandoni* hybridizes with its sister species, the smooth newt *L. vulgaris*, wherever their ranges overlap (Babik et al., 2003; Babik & Rafinski, 2004). Phylogeographic analyses of the two species have revealed a lack of concordance between the species' morphologies and mtDNA lineages, and imply widespread, multiple introgressions of *L. vulgaris* mtDNA into *L. montandoni* (Babik et al., 2005). Furthermore, haplotype sharing between *L. montandoni* and some of the geographic groups of *L. vulgaris* at several polymorphic nuclear loci indicates extensive gene flow in the nuclear genome (Babik & Nadachowska, unpublished data).

Microsatellite markers for *L. montandoni* can be used to study the genetic structure of the species at the metapopulation level, and to obtain further insights into the dynamics of interspecific hybridization. Such information is also essential in designing conservation strategies by, for example, delineating conservation units. This is important, since populations of newts are declining despite protection by law in all countries they occur (http://www.iucnredlist.org/initiatives/amphibians).

To develop microsatellite loci we used a modified protocol of Glenn & Schable (2005). Genomic DNA (2.5ug) from two individuals was digested with *Rsa*I enzyme (New England Biolabs) in a 20μL reaction volume overnight at 37°C. Thermal deactivation of the restriction enzyme was performed for 20 minutes at 80 °C. Double stranded linkers (SuperSNX) were then ligated to the DNA fragments. The ligation was performed in the presence of *Xmn*I restriction enzyme, which prevents linkers from dimerization. Linker-ligated DNA was used in DynaBead enrichment procedure. Two mixtures of 3'biotinylated oligos were used in hybridization with DNA – Mix1: $(AG)_{12}$, $(TG)_{12}$, $(AAC)_6$, $(AAG)_8$, $(AAT)_{12}$, $(ACT)_{12}$, $(ATC)_8$ and Mix2: $(AAAT)_8$, $(AACT)_8$, $(AAGT)_8$, $(ACAT)_8$, $(AGAT)_8$). To capture DNA fragments with microstatellite sequences that were complementary to the microsatellite oligos, we used DynaBeads coated with streptavidin (Dynal, Oslo, Norway) and a magnetic particle concentrator (MPC, Dynal, Oslo, Norway). We performed six wash steps (two final steps were performed using solutions heated to 50 °C) according to the protocol. We did not precipitate the enriched fragments with NaOAc/EDTA solution, but instead used MinElute columns (Qiagen) and eluted the enriched DNA in 12μL AE buffer. After the first enrichment we ran a PCR with 2 μl of eluted DNA. The following PCR protocol was used: 2.5 μL of 10xPCR buffer with $(NH_4)_2SO_4$ (Fermentas), 2.0 μL of 25mM $MgCl_2$, 0.4 μL of 10mM dNTP, 1.3 μL of SuperSNX-24F primer (100 mM), 0.2 μL of Taq (5 u/μL) polymerase (Fermentas) and $ddH_2O$ to 25 mL. The cycling schemes were as follows: 94 °C for 2 min followed by 25 cycles of 95 °C for 20s; 60 °C for 20s, 72 °C for 90s; the final extension was at 72 °C for 30min. The PCR products obtained after first enrichment were cleaned with MinElute columns (Qiagen), ligated into TOPO plasmid vector and transformed into component *E. coli* One Shot TOP10 cells (TOPO TA Cloning, Invitrogen). We used a standard blue-white screening procedure to pick the colonies with inserts and performed colony-PCR with M13 forward and reverse primers. The cycling schemes we used were as follows: 94 °C for 2 min followed by 27 cycles of 95 °C for 20s; 50 °C for 20s, 72 °C for 90s; the final extension was at 72 °C for 10 min. The PCR products from approximately 360 colonies were sequenced and screened for the presence of microsatellite repeats. However, just

*Correspondence:* Krystyna Nadachowska, Jagiellonian University, Institute of Environmental Sciences, Gronostajowa 7, 30-387, Kraków, Poland. *E-mail:* krystyna.nadachowska@uj.edu.pl

**Table 1.** Characterization of polymorphic microsatellite loci in *Lissotriton montandoni.* Pop. = populations: K1 – Krempna 1, K2 – Krempna 2, P – Predeal; $N_A$ = number of alleles observed; $H_O$ = observed heterozygosity; $H_E$ = expected heterozygosity. **P*<0.05; ****P*<0.001 after Bonferroni correction.

| Locus | Primers | Clone repeat sequence | Pop. | Size range | $N_A$ | $H_O$ | $H_E$ |
|---|---|---|---|---|---|---|---|
| *Lm_013* | F: CTTGGTTCCCAGTGAGGAGA | $(GA)_3AA(GA)_6AA(GA)_{18}CA$ | K1 | 203–221 | 7 | 0.81 | 0.80 |
| | R: GCAAGCCATCCCAAAGTAAG | $(GA)_2CA(GA)_8CA(GA)_2CA(GA)_4$ | K2 | 203–221 | 10 | 0.71 | 0.82 |
| | | | P | 217 | 1 | | |
| *Lm_488* | F: CAGGCAGGGTATTTGCGTAG | $(TATC)_{19}$ | K1 | 197–233 | 8 | 0.67 | 0.81 |
| | R: GGTCATTTCCACAACAAGCTC | | K2 | 196–229 | 10 | 0.50* | 0.83 |
| | | | P | no scoring possible | | | |
| *Lm_521* | F: CATACGGGCACTGAGGTGAT | $(GATA)_{19}(GACA)_{11}$ | K1 | 260–296 | 10 | 0.87 | 0.91 |
| | R: GCACAGACATTGATGGCAAA | | K2 | 256–292 | 11 | 0.86 | 0.91 |
| | | | P | 290–398 | 9 | 0.80 | 0.83 |
| *Lm_528* | F: CTGGCTTGAAATGCCTTCAT | $(TATC)_{16}$ | K1 | 153–198 | 9 | 0.69 | 0.88 |
| | R: AGGGCAGGGCTATACGTCTT | | K2 | 153–198 | 10 | 0.76 | 0.80 |
| | | | P | 149–186 | 8 | 0.86 | 0.80 |
| *Lm_632* | F: CAGAGCAATTTCTAGGCAAGG | $(TATC)_{10}$ | K1 | 215–239 | 6 | 0.50 | 0.73 |
| | R: GGCGCTATATCAAACTGCAA | | K2 | 219–239 | 6 | 0.67 | 0.70 |
| | | | P | 215–247 | 8 | 0.86 | 0.89 |
| *Lm_749* | F: CCATGGTGGTAGAATAAATGGAA | $(CAGA)_4(TAGA)_{13}$ | K1 | 190–245 | 9 | 0.60 | 0.81 |
| | R: AAGACCATTCTTTCTGAGGTATCC | | K2 | 190–249 | 9 | 0.57 | 0.86 |
| | | | P | 194–245 | 8 | 0.73 | 0.83 |
| *Lm_870* | F: CCACTGCTTTGTGCTGCTAC | $(ATAG)_4G(ATAG)_{24}$ | K1 | 175–206 | 8 | 0.81 | 0.84 |
| | R: TTTGTCATGGCATTTCCAAC | | K2 | 159–198 | 9 | 0.86 | 0.84 |
| | | | P | 163–220 | 13 | 0.20*** | 0.93 |

15% of screened colonies contained microsatellites (79% dinucleotide repeats, 21% tetranucleotide repeats). Thus, PCR products obtained in the first enrichment were used in the second enrichment. The PCR products obtained after the second enrichment were cleaned and cloned using the same procedure as during the first enrichment. We screened approximately 450 colonies, 85% of which contained repetitive DNA fragments (31% dinucleotide repeats, 66% tetranucleotide; three clones contained trinucleotide repeats and eight both dinucleotide and tetranucleotide repeats).

For primer design with the software Primer3Plus (Untergasser et al., 2007) we chose sequences of 40 clones containing microsatellite motifs and flanking regions long enough to design locus specific primers. To check for amplification and polymorphism of selected loci, we used the method developed by Schuelke (2000). Instead of labelling one primer per pair with expensive fluorescent dyes, we used labelled M13 forward primers. For this method the PCR is performed with three primers: the locus specific forward primer with the M13 sequence tail at its 5' end, the locus specific reverse primer and the labeled M13 forward primer. The conditions of the reaction were chosen to ensure that during the first cycles the tailed forward primer is incorporated in the PCR product sequences. Later, when the forward primer is used up, the labelled M13 primer is used in the amplifying reaction. As a result, the reaction mix contains mostly the labelled product with the M13 sequence tail. The following PCR protocol was used for 10 μl reactions: 1 × PCR buffer with $(NH_4)_2SO_4$ or 1 × *DreamTaq* buffer (Fermentas), 2.5 mM $MgCl_2$, 0.2 mM of each dNTP, 0.4 μM M13 labeled primer, 0.1 μM locus specific forward primer, 0.4 μM locus specific reverse primer and 0.5 U *Taq* or *DreamTaq* polymerase (Fermentas) and $ddH_2O$ to 10μL. The cycling scheme was as follows: 94 °C for 2.5 min followed by 34–40 cycles of 94 °C for 20s, 55 °C for 30s and 72 °C for 30s; the final extension was at 72 °C for 10 min. PCR products were electrophoresed on an ABI 3130xl Genetic Analyser with GeneScan 500 LIZ size standard (Applied Biosystems). Allele sizes were determined using GeneMapper software (Applied Biosystems). Unambiguous scoring was possible for seven polymorphic loci. Sequences of these microsatellite loci were deposited in GenBank (accession numbers GU574493–GU574499).

These seven loci were characterized in 37 individuals from Krempna (Poland, 49°29'N, 21°28'E) and in 15 individuals from Predeal (Romania, 45°49'N, 25°56'E). These populations are geographically distant (straight-line distance of approximately 550 km) and represent two different mtDNA groups (Babik et al., 2005). The samples from Krempna were collected in 2001 (K1) and 2009 (K2). Thus we decided to divide this population into two samples according to the year of collection and analysed three samples in total: 16 (K1) and 21 (K2) individuals from Krempna and 15 individuals from Predeal (P). Seven loci were polymorphic in Krempna (K1 and K2) and five loci in Predeal. One locus in Predeal could not be scored due to

**Table 2.** Cross-species amplification of 12 individuals of *Lissotriton vulgaris* from populations across the species' range. *L.v.v.* = *Lissotriton vulgaris vulgaris; L.v.g.* = *Lissotriton vulgaris greacus; L.v.k.* = *Lissotriton vulgaris kosswigi; L.v.sch.* = *Lissotriton vulgaris schmidtlerorum; L.v.m.* = *Lissotriton vulgaris meredionalis.*

| Species/country | *Lm_013* | *Lm_488* | *Lm_521* | *Lm_528* | *Lm_623* | *Lm_749* | *Lm_870* |
|---|---|---|---|---|---|---|---|
| *L.v.v.*/Croatia | + | + | + | + | – | + | – |
| *L.v.v.*/Poland | – | + | + | + | + | + | – |
| *L.v.v.*/Slovakia | + | + | + | + | + | + | – |
| *L.v.v.*/Serbia | – | + | + | + | – | + | – |
| *L.v.v.*/Bulgaria | – | + | + | – | + | + | – |
| *L.v.v.*/Hungary | + | + | + | + | + | + | – |
| *L.v.g.*/Montenegro | – | – | + | + | – | + | – |
| *L.v.g.*/Greece | – | + | + | + | + | + | – |
| *L.v.g.*/Montenegro | + | + | + | + | – | – | – |
| *L.v.k.*/Turkey | – | + | + | + | – | + | – |
| *L.v.sch.*/Turkey | – | + | + | – | – | – | + |
| *L.v.m*/Croatia | + | + | + | + | + | + | – |

its apparent duplication. The allele size ranges were similar in both populations. The only locus with almost non-overlapping allele size ranges was *Lm_521*. The size range in P was more than threefold wider than within K1 and K2.

The numbers of alleles per locus per population, as well as observed and expected heterozygosities, were calculated with Arlequin 3.1 (Excoffier et al., 2005). The number of alleles for polymorphic loci ranged from 6 to 13 and observed heterozygosities from 0.20 to 0.87 (Table 1). After Bonferroni correction the observed and expected heterozygosities differed significantly for one locus in K2 (*Lm_488*) and one locus in P (*Lm_870*). Both differences showed heterozygote deficiency, which suggests the presence of null allele(s). One of these loci (*Lm_870*) consistently did not amplify in some individuals from P. Thus we classified these individuals as null allele homozygotes and estimated the null allele frequency with GENEPOP (Rousset, 2008). The frequency of null alleles was estimated as 0.47. Two other loci (*Lm_013* in K2, *Lm_749* in K1 and K2) also showed slight heterozygote deficiency, though non-significant after Bonferroni correction. No significant linkage disequilibrium was detected between any pair of loci.

All loci were tested for cross-amplification in 12 individuals of *Lissotriton vulgaris* from populations across the species' range. We used the same PCR protocol as for *L. montandoni* samples. *L. vulgaris* sampling reflected information on the distribution of the morphologically distinguishable subspecies (Schmidtler & Franzen, 2004), mtDNA lineages (Babik et al., 2005) and nuclear sequence variation (Babik & Nadachowska, unpublished). The sample included individuals from five subspecies: *L. v. vulgaris* (six populations, *L.v.v.*), *L. v. graecus* (three populations, *L.v.g.*), *L. v. kosswigi* (one population, *L.v.k.*), *L. v. schmidtlerorum* (one population, *L.v.sch.*) and *L. v. meridionalis* (one population, *L.v.m.*). The amplification success was variable (Table 2). Some of the loci were amplified just in a subset of tested populations including also differential amplification across populations of the same subspecies. The only locus that amplified in all 12 individuals was *Lm_521*. We observed a similar pattern when we tested microsatellite primers developed for the Greek smooth newt by Sotiropoulos et al. (2009) on other *L. vulgaris* subspecies. We did not score variation in the amplified loci.

Our cross-amplification experiment shows that the application of the newly developed markers across subspecies of the smooth newt may be limited, which is in accordance with the findings of Johanet et al. (2009), who also found little cross-species amplification success for another panel of microsatellites developed for species of *Lissotriton*. The cross-species amplification problems may have multiple causes. First, microsatellites are usually found in noncoding regions where substitution rates are typically high. Thus, developing markers that can be used in species that diverged long time ago can be problematic (Primmer & Merila, 2002; Zane et al., 2002). The divergence time between *L. montandoni* and *L. vulgaris* has been estimated at 3–5 Mya (Babik et al., 2005). Therefore it is possible that primers would cross-amplify mainly in genomic regions that introgress between species, and it is known that only some geographic groups of the smooth newt, including the Romanian population used in the study (Babik et al., 2005; Babik & Nadachowska, unpublished), are involved in hybridization with the Carpathian newt (Babik et al., 2005). However, cross-species amplification success was variable across all subspecies including groups with high levels of introgression (nominative subspecies of *L. vulgaris*) and other subspecies. Second, many amphibians form metapopulations (Marsh & Trenham, 2001) and are known to exhibit a high degree of spatial structure (Newman & Squire, 2001). Subdivision may persist for a long evolutionary time (Kozak et al., 2006), which is also the case in *L. vulgaris* and *L. montandoni* (Babik et al., 2005; Nadachowska & Babik, 2009). Differences in the genome accumulated through time in structured populations may hamper amplification of microsatellite loci due to mutations at primer binding sites. Third, although there is a positive correlation between genome size and frequency of microsatellite loci, genome size can

be negatively correlated with successful amplification of these markers (Garner, 2002) and many urodeles, including the Carpathian newt (estimated haploid genome size 27–29 pg), have large genomes (Gregory, 2005).

*Acknowledgements*. This work was supported by the Foundation for Polish Science, professor subsidy 9/2008 to Prof. Jacek Radwan.

## REFERENCES

Babik, W., Branicki, W., Crnobrnja-Isailovic, J., Cogalniceanu, D., Sas, I., Olgun, K., Poyarkov, N.A., Garcia-Paris, M. & Arntzen, J.W. (2005). Phylogeography of two European newt species – discordance between mtDNA and morphology. *Molecular Ecology* 14, 2475–2491.

Babik, W. & Rafinski, J. (2004). Relationship between morphometric and genetic variation in pure and hybrid populations of the smooth and Montandon's newt (*Triturus vulgaris* and *T. montandoni*). *Journal of Zoology* 262, 135–143.

Babik, W., Szymura, J.M. & Rafinski, J. (2003). Nuclear markers, mitochondrial DNA and male secondary sexual traits variation in a newt hybrid zone (*Triturus vulgaris* x *T.montandoni*). *Molecular Ecology* 12, 1913–1930.

Excoffier, L., Laval, G. & Schneider, S. (2005). Arlequin (version 3.0): an integrated software package for population genetics data analysis. *Evolutionary Bioinformatics* 1, 47–50.

Garner, T.W.J. (2002). Genome size and microsatellites: the effect of nuclear size on amplification potential. *Genome* 45, 212–215.

Glenn, T.C. & Schable, N.A. (2005). Isolating microsatellite DNA loci. In *Molecular Evolution: Producing the Biochemical Data, Part B*, 202–222. Zimmer. E.A. & Roalson, E. (eds). San Diego, USA: Academic Press.

Gregory, T.R. (2005). *Animal Genome Size Database*. http://www.genomesize.com.

Johanet, A., Picard, D., Garner, T.W.J., Dawson, D.A., Morales-Hojas, R., Jehle, R., Peltier, D. & Lemaire, C. (2009). Characterization of microstellite loci in two closely related *Lissotriton* newt species. *Conservation Genetics* 10, 1903–1906.

Kozak, K.H., Weisrock, D.W. & Larson, A. (2006). Rapid lineage accumulation in a non-adaptive radiation: phylogenetic analysis of diversification rates in eastern North American woodland salamanders (Plethodontidae: *Plethodon*). *Proceedings of the Royal Society B* 273, 539–546.

Marsh, D.M. & Trenham, P.C. (2001). Metapopulation dynamics and amphibian conservation. *Conservation Biology* 15, 40–49.

Nadachowska, K. & Babik, W. (2009). Divergence in the face of gene flow: the case of two newts (Amphibia: Salamandridae). *Molecular Biology and Evolution* 26, 829–841.

Newman, R.A. & Squire, T. (2001). Microsatellite variation and fine-scale population structure in the wood frog (*Rana sylvatica*). *Molecular Ecology* 10, 1087–1100.

Primmer, C.R. & Merila, J. (2002). A low rate of cross-species microsatellite amplification success in ranid frogs. *Conservation Genetics* 3, 445–449.

Rousset, F. (2008). GENEPOP '007: a complete re-implementation of the GENEPOP software for Windows and Linux. *Molecular Ecology Resources* 8, 103–106.

Schmidtler, J.F. & Frantzen, M. (2004). *Triturus vulgaris* (Linnaeus, 1758) – Teichmolch. In *Handbuch der Reptilen und Amphibien Europas, Vol. Band 4/IIB*, 847–967.

Schuelke, M. (2000). An economic method for the fluorescent labeling of PCR fragments. *Nature Biotechnology* 18, 233–234.

Sotiropoulos, K., Eleftherakos, K., Tsaparis, D., Kasapidis, P., Magoulas, A. & Legakis, A. (2009). New polymorphic microsatellite loci for the Greek smooth newt, *Lissotriton vulgaris graecus*, and their utility in the nominotypical subspecies. *Molecular Ecology Resources* 9, 292–295.

Untergasser, A., Nijveen, H., Rao, X., Bisseling, T., Geurts, R. & Leunissen, J.A.M. (2007). Primer3Plus, an enhanced web interface to Primer3. *Nucleic Acids Research* 35, W71–W74.

Zane, L., Bargelloni, L. & Patarnello, T. (2002). Strategies for microsatellite isolation: a review. *Molecular Ecology* 11, 1–16.

Zavadil, V., Piálek, J. & Dandová, R. (2003) *Triturus montandoni* (Boulenger, 1880) – Karpatenmolch. In *Handbuch der Reptilen und Amphibien Europass, Vol. Band 4/IIB*, 657–706. Böhme, W. (ed.). Wiebelsheim: Aula.

*Accepted: 11 January 2010*