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Genetic variation of *Lissotriton montandoni* from the eastern part of the Slovak Carpathians

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Abstract. This study investigated the extent of genetic variation in *Lissotriton montandoni* individuals collected from three locations in the eastern Slovak Carpathians using microsatellite DNA markers. The genetic characteristics of these microsatellite loci were confirmed to be suitable for molecular genetic studies in *L. montandoni*, as indicated by high polymorphic information content values. Furthermore, a high level of genetic variation was detected in this endemic species of amphibian. The fixation index values suggested minimal differentiation among the three analyzed subpopulations, with only 1% of the total genetic variation occurring between subpopulations, 3% between individuals, and 96% within individuals. The presence of a high number of alleles at the same chromosomal loci contributes to genetic variation across the entire population, which is beneficial and essential for the adaptation of both individuals and the population as a whole to current and future environmental changes.

Keywords: Carpathian Newt, Eastern Carpathians, genetic diversity, microsatellite

Introduction

Genetic diversity is a key component of biodiversity playing a fundamental role in the long-term survival and adaptability of species and in maintaining healthy populations and ecosystems. It is defined as the variation in genes within and between populations of a given species. Diversity needs to be recognised, and its extent and trends described. One of the species for which such a study is necessary is *Lissotriton montandoni* (Boulenger 1880), commonly known as the Carpathian Newt. This species is an endemic amphibian species of the Eastern Carpathians and the easternmost Sudetes. It is found in the Czech Republic, Poland, Romania, Slovakia, and Ukraine and is listed in the International Union for Conservation of Nature (IUCN) Red List of Threatened Species. *L. montandoni* is here classified as "least concern", indicating a stable population and low risk of extinction. However, this does not diminish its conservation importance within the European Union (EU). Under EU legislation, *L. montandoni* is listed in Annex IV of the Habitats Directive (92/43/EEC), which identifies animal and plant species of community interest in need of strict protection. This listing mandates that EU member states implement measures to conserve the species and its habitats. Additionally, the species is included in the Natura 2000 network, a network of protected areas across the EU aimed at conserving biodiversity. The primary regions of its distribution and genetic diversity has been documented in Romania and Ukraine (Zieliński *et al.*, 2014). Genetic variation within populations is crucial for the adaptation of individuals and the survival of the whole population in response to current and upcoming environmental changes. A higher level of genetic variation enhances a population's adaptive capacity. This variation is assessed not only through phenotypic traits but also through genomic diversity in both coding and non-coding DNA sequences. An increasing number of different alleles at the same chromosomal locus contributes to greater genetic variation within the population. In *L. montandoni*, genetic variation is further influenced by sympatry with *Lissotriton vulgaris*. These two species can interbreed, forming hybrids in areas where their ranges overlap (Kotlík and Zavadil, 1999; Gherghel *et al.*, 2012). The assessment of the extent of genetic variation requires highly informative genetic markers, such as microsatellite markers. Previous studies have documented allelic diversity at microsatellite nuclear DNA loci in *L. montandoni* (Johanet *et al.*, 2009; Zieliński *et al.*, 2013). However, the populations of *L. montandoni* in the northwestern Carpathians of Slovakia remains poorly studied. So far, only two populations have been compared from Slovakia, and even then only based on a few morphological features of the newt (Kniha *et al.*, 2013). A study that analyzed the genetic variation in *L. montandoni* populations, also by application of DNA analyses, has not been carried out in Slovakia to date.

The aim of this study was therefore to use specific microsatellite markers to analyze genetic variation within and between subpopulations of this amphibian in areas known to be its habitat.

Materials and methods

Samples of Carpathian newt were collected in two locations of the Eastern Tatras (Tatranská Javorina and Tatranská Kotlina) and one from the Poloniny National Park (Fig. 1).

Figure 1. Sampling localities of *L. montandoni* (red diamonds). Two on the top – Tatranská Javorina and Tatranská Kotlina, one on the right side – Poloniny.

Genomic DNA was extracted from tail samples (1 mm^2) using the Wizard® SV Genomic DNA Purification System (Promega Corp., Madison, USA). The sample sizes were as follows: five individuals from Tatranská Javorina, three from Tatranská Kotlina, and seventeen from Poloniny. Microsatellite loci (Lm_488, Lm_521, Lm_528, Lm_632, Lm_749, and Lm_870) were amplified using primer pairs developed by Nadachowska *et al.* (2010). Forward primers were fluorescently labelled at the 5' end with ATTO550, ATTO565, FAM, or HEX. Polymerase chain reactions (PCR) were performed in 20 µl volumes containing 50 ng of DNA, 10 pmol $µl^{-1}$ of both primers, 10 mM dNTPs, 4 µl of Q-solution (Qiagen N.V., Venlo, The Netherlands), 2 µl of 10x PCR buffer, 2 mM MgCl2, 1 U of HotStar Taq DNA polymerase (Qiagen N.V., Venlo, The Netherlands), and

water to the final volume. The thermal cycling profile of the PCR consisted of an initial denaturation at 95 °C for 15 min, followed by 29 cycles of 94 °C for 30 s, 55 °C (for Lm_521, Lm_528, Lm_632, Lm_749) or 57 °C (for Lm_488, Lm_870) for 60 s. This was followed by a final extension step was performed at 72 °C for 90 s. Subsequently, the PCR products were analyzed by capillary electrophoresis using the automated ABI PRISM® 3100-Avant Genetic Analyzer (Applied Biosystems, Waltham, USA). The fragment sizes were determined using the GeneMapper™ software (Thermo Fisher Scientific, Waltham, USA). The polymorphic information content (PIC) was calculated using Cervus software version 3.0.7 (Kalinowski *et al.*, 2007). The genetic parameters evaluated included the number of alleles per locus (N_a), the number of effective alleles per locus (N_e), observed heterozygosity, expected heterozygosity, and the fixation index (F_{ST}). These were analyzed using GenAIEx v 6.5 software (Peakall and Smouse, 2012). Cluster analysis was conducted using two-way visualisation with UPGMA clustering and Bray-Curtis similarity in Past 4.17c (Hammer *et al.*, 2001).

Results

The first part of the results presents the parameters of the microsatellite markers (loci) used to assess genetic variation in the collected *L. montandoni* samples (Tab. 1).

Table 1. Genetic parameters of six microsatellite loci analyzed.

Locus	N	N_a	N_e	H_o	H_e	F_{ST}	PIC	I
Lm521	25	16	12.500	0.960	0.920	-0.043	0.914	2.637
Lm528	25	12	7.440	0.920	0.866	-0.063	0.854	2.249
Lm749	25	10	4.613	0.640	0.783	0.183	0.759	1.819
Lm632	25	8	4.223	0.840	0.763	-0.101	0.732	1.675
Lm488	23	10	5.038	0.652	0.802	0.186	0.782	1.920
Lm870	25	11	6.906	0.960	0.855	-0.123	0.840	2.111

(N) number of samples; (N_a) number of alleles; (N_e) number of effective alleles; (H_o) observed heterozygosity; (H_e) expected heterozygosity; (F_{ST}) fixation index; (PIC) polymorphic information content; (I) Shannon's information index.

A total of 67 alleles (8-16 per locus) were identified across 25 individuals from the three sampling sites. The number of effective alleles per locus ranged from 4.220 to 12.500, with the highest values at locus Lm521 (16 alleles and 12.50 effective alleles). Expected heterozygosity ranged from 0.763 to 0.920, while observed heterozygosity varied from 0.640 to 0.960. The PIC values

ranged from 0.732 to 0.914 and Shannon's information index (1.675-2.637) indicate the informativeness and high genetic diversity of the microsatellite markers used in this study for analyzing genetic variation. Additionally, these findings underscore a substantial degree of genetic diversity in *L. montandoni*, even when considering the relatively modest sample size from the three Slovakian locations.

The second part of the results compares the genetic variation of *L. montandoni* across the three study locations (Tab. 2).

Table 2. Genetic parameters of analyzed populations of *L. montandoni* from three locations.

Location	Locus	N	N_a	N_e	H_o	H_e	F_{ST}	I
Poloniny	Lm521	17	15.000	11.796	0.941	0.915	-0.028	2.581
	Lm528	17	11.000	7.225	0.941	0.862	-0.092	2.190
	Lm749	17	8.000	4.158	0.529	0.760	0.303	1.693
	Lm632	17	7.000	4.379	0.882	0.772	-0.143	1.658
	Lm488	15	8.000	4.412	0.600	0.773	0.224	1.752
	Lm870	17	9.000	6.568	0.941	0.848	-0.110	2.003
Tatranská Kotlina	Lm521	3	4.000	3.600	1.000	0.722	-0.385	1.330
	Lm528	3	5.000	4.500	1.000	0.778	-0.286	1.561
	Lm749	3	3.000	2.571	0.667	0.611	-0.091	1.011
	Lm632	3	3.000	2.000	0.667	0.500	-0.333	0.868
	Lm488	3	3.000	2.571	0.333	0.611	0.455	1.011
	Lm870	3	5.000	4.500	1.000	0.778	-0.286	1.561
Tatranská Javorina	Lm521	5	7.000	6.250	1.000	0.840	-0.190	1.887
	Lm528	5	6.000	4.167	0.800	0.760	-0.053	1.609
	Lm749	5	5.000	3.125	1.000	0.680	-0.471	1.359
	Lm632	5	6.000	3.333	0.800	0.700	-0.143	1.498
	Lm488	5	7.000	6.250	1.000	0.840	-0.190	1.887
	Lm870	5	7.000	5.556	1.000	0.820	-0.220	1.834

(N) number of samples; (N_a) number of alleles; (N_e) number of effective alleles; (H_o) observed heterozygosity; (H_e) expected heterozygosity; (F_{ST}) fixation index; (I) Shannon's information index.

The mean number of alleles and the number of effective alleles per individual was 16.67 (N_a) and 6.42 (N_e) in Poloniny, 6.33 (N_a) and 4.78 (N_e) in Tatranská Javorina, and 3.83 (N_a) and 3.29 (N_e) in Tatranská Kotlina. The mean expected heterozygosity and observed heterozygosity were 0.822 (H_e) and

0.806 (H_o) in Poloniny, 0.667 (H_e) and 0.778 (H_o) in Tatranská Kotlina, and 0.773 (H_e) and 0.933 (H_o) in Tatranská Javorina. The fixation index (F_{ST}) values ranged from 0.00 to 0.455, indicating minimal differentiation among the three subpopulations and suggesting a high degree of genetic similarity. The analysis of molecular variation revealed that only 1% of the total genetic variation was observed among subpopulations, 3% among individuals, and a significant 96% was within individuals.

The cluster analysis (Fig. 2) generated three key outcomes. Firstly, the *L. montandoni* exhibited a high level of genetic variation, as evidenced by the fact that no two individuals shared an identical microsatellite profile. Secondly, the absence of significant differentiation among the three subpopulations suggests a high degree of genetic overlap among individuals from all three localities (Fig. 2). The relative short geographical distances between the collection sites (approximately 170 kilometers apart by air) do not appear to be sufficient for genetic differentiation. In contrast, differentiation between "southern" and "northern" populations of *L. montandoni* has only been observed when analyzing individuals from localities spanning the entire Carpathian arc, which extends up to approximately 1,000 kilometers (Zieliński *et al.*, 2014). Thirdly, two individuals from Poloniny (marked in red diamond on the right side of Fig. 1) were genetically distinct from all others. This distinction is attributed to the presence of null alleles at the Lm488 locus in a homozygous state.

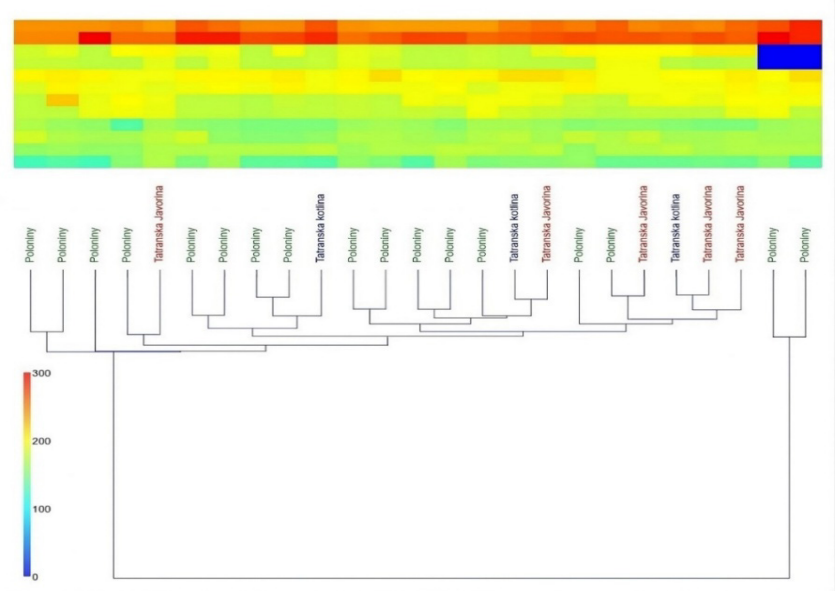

Figure 2. Differentiation of *L. montandoni* individuals and subpopulations by UPGMA clustering.

Discussion

The microsatellite markers used in this study confirmed their effectiveness in detecting high genetic variation in *L. montandoni* populations. The number of alleles per locus (8 – 16) observed in the three studied subpopulations from a relatively small area of the Slovak Eastern Carpathians was comparable to the findings of Nadachowska *et al.* (2010), who analyzed the same microsatellite loci in populations from two geographically distant locations (approximately 540 kilometers apart by air), one in Poland and the other in Romania. However, the Slovak subpopulations exhibited higher observed heterozygosity (H_o) values, ranging from 0.64 to 0.96, compared to 0.20 to 0.87 in the earlier study.

The contrasting mean number of alleles (N_a) and effective alleles (N_e) per individual across the three locations is intriguing. Poloniny displayed considerably lower values compared to Tatranská Javorina and Tatranská Kotlina. This may suggest historical demographic factors, population bottlenecks, or varying levels of gene flow affecting the Poloniny population. Although F_{ST} values indicate minimal differentiation, the slightly higher expected and observed heterozygosity (H_e and H_o) values in Poloniny compared to Tatranská Kotlina are noteworthy, especially considering the lower overall number of alleles. This could reflect a more even allele distribution in Poloniny population, despite reduced overall diversity. The findings that 96% of the genetic variation occurs within individuals underscores the high level of individual genetic diversity in *L. montandoni* across the Slovak populations. This support the idea of significant gene flow or a recent common ancestry among the populations. Additionally, the fact that no two individuals share identical microsatellite profile further emphasized the genetic richness present in this relatively small sample size, allowing insight into the genetic structure of the species in this region.

Of particular interest is the identification of two individuals from Poloniny that were genetically distinct from all others due to homozygosity for null alleles at the Lm488 locus. This highlights the potential impact of technical artifacts, such as null alleles, on genetic analyses and underscores the importance of accounting for such factors during data interpretation.

The absence of clear clustering by sampling site (Figure 2) visually supports the low F_{ST} values and the high degree of genetic overlap among the subpopulations. The relatively short geographical distance (170 km) between the study sites appears insufficient to drive significant genetic differentiation, at least based on the resolution provided by microsatellite markers. A comparison with broader-scale studies, such asthat by Zieliński *et al.* (2014) across the Carpathian arc, provides useful context and suggests that geographical range becomes a more significant barrier to gene flow only over larger spatial scales.

This study provides foundational insights into the genetic diversity and population structure of *Lissotriton montandoni* in three Slovak localities. However, a significant limitation is the very small sample size from the Poloniny population (n = 3), which may reduce the reliability of estimates for allelic diversity and population structure at this site. Small sample sizes can increase the likelihood of sampling bias, underestimate genetic variation, and limit the ability to detect rare alleles. Consequently, the observed lower allelic diversity in Poloniny should be interpreted with caution. Adequate sample size is important in genetic analyses. Although small sample sizes may be considered low for some population genetic analyses, maximizing the number of markers may be beneficial for robust landscape genetic inferences in *L. vulgaris* populations (Zieliński *et al.*, 2014) or observed strenght of spatial genetic patterns (Landguth *et al.*, 2011). Despite this limitation, the dataset offers valuable baseline information that can inform future conservation strategies and serve as a reference for comparative studies across the species' range. Overall, the current study highlights important directions for continued investigation into the population genetics of *L. montandoni* and underscores the need for comprehensive sampling across its distribution.

Conclusions

The study yielded results indicating a high degree of genetic diversity within *L. montandoni* subpopulations from Slovakia and revealed minimal genetic structuring among the three sampled sites. These results have important implications for conservation strategies, suggesting that even relatively small populations can harbor considerable genetic resources and emphasizing the importance of maintaining connectivity among habitats to preserve this diversity.

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