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Genetic differentiation over a small spatial scale in the smooth newt (*Lissotriton vulgaris*)

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Understanding the distribution of genetic variation is central for both population biology and conservation genetics. Genetic population structure can be primarily affected by the species' dispersal ability, which is assumed to be limited in many amphibians. In this study, we estimated allelic differentiation metrics and F_{ST} indices to investigate genetic variation among natural breeding ponds of smooth newts (*Lissotriton vulgaris*) over a small spatial scale. Based on six microsatellite loci, we found a small, but significant allelic differentiation among clusters of natural breeding ponds (i.e. 'local regions'), which result was in line with the calculation of corresponding hierarchical F_{ST} values. Analysis of molecular variance also indicated significant between-region variation in the study area. Pairwise estimations showed that only the furthermost regions differed from each other in both differentiation measures, but this difference was not attributable to geographic distances between ponds. Our results provide evidence that hierarchical genetic structure can be characteristic to breeding ponds of smooth newts on a small spatial scale in their natural breeding habitat, but dispersal distance may be less limited than previously thought in these philopatric caudates.

Keywords: genetic differentiation, natural habitats, isolation by distance, philopatric species, hierarchical diversity partitioning, fixation indices, Shannon entropy, *Lissotriton vulgaris*

INTRODUCTION

Genetic variation forms the basis for various micro-evolutionary processes and has a fundamental role in the long-term persistence of populations and species (Hoffmann & Willi, 2008). Because of that, investigating the distribution of genetic variation and prevalent genetic structure is important from both theoretical and applied perspectives (Holderegger et al., 2006; Petit et al., 1998; Smouse & Peakall, 1999). Studying genetic diversity in natural populations, for instance, may reveal those spatial characteristics that facilitate gene flow and influence the structural properties of migration/dispersal networks, and help to identify areas or populations that should be prioritised when allocating conservation efforts (Emel et al., 2019; Lesbarreres et al., 2006). Amphibians are among the most threatened vertebrate taxa worldwide (Arntzen et al., 2017; Powers & Jetz, 2019), so more and more investigations are being carried out on amphibian species to scrutinise how landscape characteristics affect genetic differentiation between breeding populations at various spatial scales (Atlas & Fu, 2019; Almeida-Gomes & Rocha, 2014; Luqman et al., 2018). Other studies utilise genetic data to test whether amphibian populations actually exhibit metapopulation dynamics as a priori expected in many species; this knowledge is crucial for the appropriate conservation management of breeding locations (Marsh & Trenham, 2001; Watts et al., 2015; Billerman et al., 2019).

The smooth newt is one of the most widespread newt species in Europe (Arntzen et al., 2009), and a popular subject for the study of developmental plasticity (e.g. Martin et al., 2016; Tóth & Hettyey, 2018), mate choice (e.g. Secondi & Théry, 2014; Secondi et al., 2015) and hybridisation between sister species (e.g. Zieliński et al., 2019; Niedzicka et al., 2020). Based on findings of conventional mark-recapture studies, smooth newts are regarded to be highly philopatric, with females usually returning to their natal pond to reproduce (Bell, 1977), and adults and juveniles having very short dispersal distances (ranging between 50-182 metres; Griffiths, 1984; Warwick, 1949; Bell, 1977; Dolmen, 1981).

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However, such poor dispersing ability may be unrealistic for many landscapes and seemingly contradicts the broad distribution of the species. Dispersal characteristics have important consequences on the spatial distribution of genetic variation. If dispersal is as limited as implied by the above empirical findings, isolation by distance (IBD) can be expected to shape genetic variation even on small spatial scales. IBD may lead to higher genetic similarity between pairs of populations that are close to each other compared to populations that are farther away from each other, without any selective advantage of such patterns (Meirmains, 2012; Diniz-Filho et al., 2013). On the other hand, limited dispersal may also increase differentiation in mean phenotype among populations, facilitating local adaptation (Blanquart et al., 2012; Arendt, 2015), or increase the risk of genetic drift that could, in some instances, counteract adaptation (Frankham et al., 2010). A more recent study based on the analysis of both capture-mark-recapture and genetic data did not support the observation of such limited dispersal in this species, however. In an agricultural area of small spatial scale (with 270-1800 metres between-pond distances), Schmidt et al. (2006) found overall low genetic differentiation in allozyme loci across five breeding population of smooth newts. They also showed that significant genetic differentiation was present only between some ponds that were at least 930 metres apart, but adult migration and gene flow occurred between other ponds that were even farther away. Similarly, low differentiation patterns can be expected in natural wooded landscapes, especially as shelters for all terrestrial stages, humidity at the ground level and the presence of temporary, small surface waters in natural habitats may increase survival probability and facilitate juvenile dispersal and/or between-pond migration of breeding adults. Furthermore, juveniles might disperse to much greater distances from the breeding ponds compared to adults (with a maximum of ca. 1000 metres; Müllner, 2001; Sinsch & Kirst, 2015).

In this study, we characterised the distribution of genetic variation between breeding ponds of smooth newts (*Lissotriton vulgaris*) at a small spatial scale. We sampled adults from 10 water bodies located in an approx. 10 km^2 area of natural landscape during their reproductive period, and measured genetic diversity and estimated neutral genetic differentiation on the pond and local region (i.e. groups of ponds) levels using six microsatellite loci. For comparison, we also calculated F_{st} values on the same hierarchical levels (Yang, 1998; Goudet, 2005; 2007). In accordance with the findings of Schmidt et al. (2006), we predicted that genetic differentiation would be observed, if at all, only at the local region level and isolation by distance would be negligible in the distribution of genetic variation within local regions on the studied spatial scale.

MATERIALS AND METHODS

Study area

The study area is located in the north-eastern part of the Pilis-Visegrád Mountains, Hungary, and belongs to the operational area of the Danube-Ipoly National Park (Fig. 1, Table S1). Smooth newts regularly breed in 10 permanent and semi-permanent ponds located on an approx. 10 km^2 area of deciduous forests and natural clearings (Tóth et al., 2011; Tóth, 2015; Bókony et al., 2016). The area is also characterised by the presence of two secondary asphalt roads, as well as a few forestry dirt roads, and several temporary and semi-permanent watercourses. However, there were no other potential breeding ponds within the study area in the studied years to the best of our knowledge. The altitude of the sampled ponds varies between 254 and 538 meters above sea level. Ponds were grouped into three 'local regions' ('Upper' (U) region: P1, P2, P3, P4, P5; 'Middle' (M) region: P6, P7; 'Lower' (L) region: P8, P9, P10) based on the spatial distance between them and the estimated maximal dispersal distance of juvenile smooth newts (Fig. 1). Within-region distance (measured as path lengths taking the variation in altitude between ponds also into account) ranged between 55.1 and 866.9 metres, while the distance among ponds belonging to different regions varied between 1320.4 and 3566.4 metres (Table S1; Fig. S1).

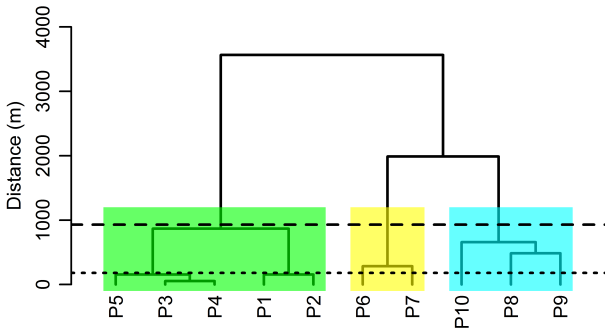

Figure 1. Dendrogram from the hierarchical clustering of ponds based on between-pond distances. Dashed line indicates the threshold value of 930 metres, which corresponds to the minimum distance between breeding sites of smooth newts that were found to be genetically differentiated by Schmidt et al. (2006). We used this value to define local regions in our study: ponds belonging to the 'Upper' local region are shown with a green background, ponds belonging to the 'Middle' local region are marked with a yellow background, and ponds belonging to the 'Lower' local region are illustrated with a cyan background, respectively. Dotted line shows the largest reported dispersal distance of 182 metres reviewed in Smith & Green (2005).

Sample collection and DNA extraction

We captured smooth newts during their breeding season (March-April) using underwater traps and by dip-netting in 2014 and 2015. In 2014, gravid females were collected from ponds P3, P6, P8 and P9 (Table S1; Tóth, 2015), while in 2015, both females and males were caught from all 10 ponds. We brought the animals to

the laboratory in individual plastic boxes appropriate for transportation. We anesthetised the individuals by inserting them into a 0.2 % solution of MS-222 (CAS: 886-86-2, Sigma-Aldrich Co., USA), then we collected swab samples from them using buccal swabs (Goldberg, Kaplan & Schwalbe, 2003; Pidancier, Miquel & Miaud, 2003). Animals were anesthetised in order to reduce the risk of injury during swab sampling and to take photographs of the collected individuals. Samples were stored at 4 °C until DNA extraction. DNA was extracted and purified using the QIAamp DNA Investigator Kit (QIAGEN N.V., Venlo, The Netherlands), following the protocol of the manufacturer. The concentration and purity of extracted DNA was estimated using NanoDrop. We evaluated fifteen microsatellite loci that were previously described and used for population genetics analyses in this species: Tv3Ca9, Tv4Ca9, Tv5Ca13 (Johanet et al., 2009), Lm_749, Lm_528, Lm_632, Lm_521, Lm_013, Lm_870, Lm_488 (Nadachowska, Flis and Babik, 2010), Lm_346, Lm_AHNC3 (Nadachowska-Brzyska et al., 2012), Lm_ZN5, Lm_TDP, Lm_8BH (Zieliński et al., 2013). Primer synthesis, multiplex labeling PCR and fragment analyses were performed by Biomi Ltd. (Gödöllő, Hungary) following the protocol reported in Zieliński et al. (2013). The PCR products were electrophoresed on a 3130xl Genetic Analyzer with GeneScan 500 LIZ size standard, and GeneMarker v2.7.0 (fully functional validation version; SoftGenetics, State College, PA, USA) was used for manual allele scoring. Six out of the 15 evaluated loci (Lm_013, Lm_528, Lm_870, Lm_488, Lm_ZN5, Lm_TDP) yielded very low or ambiguous peaks (likely due to the low quality/quantity of DNA obtained from the swabs samples or suboptimal ratio of microsatellite PCR products in the product mix, relative to each other), and therefore were discarded from the study.

Genetic and statistical analysis

We used the tandem 1.09 software (Matschiner & Salzburger, 2009) for the automatic binning of microsatellite allele sizes in the nine loci that produced reliable amplifications. As we found higher average rounding error in terms of allele size (i.e. many detected fragment lengths could not be classified unambiguously as one or another allele defined in base pairs) compared to the recommended threshold value in additional three loci (Lm_521, Lm_632 and Tv3Ca9), all further analyses were conducted using only the remaining six loci (Table S2). This way, we could ensure that ambiguously identified allele sizes would not bias our estimations of genetic differentiation between ponds. We checked for stuttering and null alleles using Micro-Checker 2.2.3 (Van Oosterhout et al., 2004). Probability of identity (P_{ID}) of increasing numbers of loci and pairwise estimators of relatedness (Lynch & Ritland, 1999) between individuals were calculated with Genalex 6.503 (Peakall & Smouse, 2012). The number of alleles (A), allelic richness (Ar; calculated for each locus as the number of alleles divided by the number of samples without missing data at that locus), observed and expected heterozygosity (H_o and H_e, respectively) were computed using the 'strataG' R package (Archer et al., 2016). Tests of Hardy-Weinberg equilibrium (HWE) and tests for linkage disequilibrium (LD) were calculated in each sampled breeding pond using Genepop 4.7.0 (Rousset, 2008); we applied the Bonferroni procedure to control for type I error.

Genetic diversity in the hierarchically structured ecosystem-region-pond system (i.e. all sampled ponds, groups of ponds and individual ponds, respectively) was decomposed based on the calculation of Hill numbers of order 1, which weights all elements in proportion to their frequency and leads to diversity measures based on Shannon's entropy (Jost, 2006; 2008; Jost et al., 2018; Gaggiotti et al., 2018). Gamma (on the ecosystem level), alpha and beta (both on the local region and pond levels) diversity components were calculated separately for each locus using the 'iDIP' function in the supplementary R script published by Gaggiotti et al. (2018). As beta diversities depend on both the actual number of local regions/ponds and their weights (number of individuals sampled within each region/pond), we also calculated normalised differentiation indices (ΔD) to quantify compositional differentiation at given hierarchical levels. We used the average values of these measures over the six loci to characterise region- and pond-level allelic differentiation as in Gaggiotti et al. (2018). We also calculated hierarchical F_{ST} values and F_{ST} analogues as "fixation" measures; such metrics, being sensitive only to demographic variables, reflect the degree of completion toward fixation and not the actual degree of differentiation of allele frequencies between populations (Jost et al., 2018). We computed hierarchical F_{ST} values on the local region and pond levels for comparison using the 'hierfstat' R package (Goudet & Jombart, 2015). We used permutation tests with 9999 iterations to examine if the observed differentiation measures were different from the ones estimated from permutation distributions, which were created by reshuffling individuals among ponds and local regions. In the case of F_{ST}, the best statistics to test for differentiation is proposed to be the likelihood ratio *G*-statistics (Goudet et al., 1996; Goudet, 2005; De Meeûs & Goudet, 2007). We used the in-built 'test.g' function of the 'hierfstat' package (which is equivalent to the 'test.between' function with the level of randomisation set to the level of individuals) to test the significance of given hierarchical levels on genetic differentiation. In order to uncover which local regions differed from each other, we estimated region-level differentiation measures between pairs of local regions, and compared the observed values to corresponding permutation distributions. One-tailed conservative *P*-values were calculated as $(b+1)/(m+1)$, where b is the number of permutation test statistics (either G^* [i.e. multilocus *G*-statistics] or ΔD values) equal or greater than the observed ones, and m is the number of iterations (Phipson & Smyth, 2010). Analysis of molecular variance (AMOVA) was also used to compute the ratio of variance components obtained from a matrix of squared Euclidean distances between pairs of individuals; this analysis represents an alternative computational method to test possible differences in a nearness to fixation measure (Φ

statistics) among different hierarchical levels (Excoffier et al., 1992; Meirmans, 2006). AMOVA components were tested for significance with 9999 permutations. We calculated Nei's pairwise F_{ST} values between ponds, and tested for IBD by investigating the correlation between linearised pairwise F_{ST} measures (Rousset, 1997) and the logarithm of geographical distances using Mantel's test (Jensen et al., 2005). For these latter tests, we used the relevant functions of the 'poppr' (Zamvar et al., 2014) and 'vegan' (Oksanen et al., 2019) R packages, respectively. We also estimated the number of genetically different clusters of ponds by conducting a Bayesian cluster analysis in GENELAND (Guillot et al., 2005, 2012). We used spatial explicit Bayesian model based on simulations of microsatellite data and geographic information on pond locations; MCMC simulations consisted of 2000000 iterations with a thinning of 1000 correlated allele frequencies and a burn-in of 200. We performed 10 independent runs and based our inference on the run giving the highest average posterior probability as suggested in the GENELAND manual. Descriptive statistics and differentiation measures were calculated and statistical tests (except the tests of HWE and for LD) were performed in R 3.6.1 (R Core Team, 2019).

RESULTS

We found no evidence of scoring error due to stuttering, large allele dropout or null alleles in the six loci. Probability of identity was estimated at 1.765×10-7, being threefold below the conservative threshold of $P_{ID} \leq$ 0.0001 (Waits et al., 2001), whereas the average pairwise relatedness among the sampled individuals was -0.005 ± 0.071 (mean ± SD) with a maximum value of 0.415. Thus, we concluded that we sampled the individuals only once and our dataset was adequate for estimating genetic diversity in the study area. The number of alleles per locus ranged from 5 (Tv4Ca9) to 27 (Lm_749) with expected heterozygosity values in the range of 0.622–0.936 (Table S2). After applying Bonferroni correction for multiple tests, we detected no significant LD for any locus pair or deviation from HWE for any loci in any of the sampled ponds.

The effective numbers of alleles calculated for each locus are shown in Table 1. Allelic differentiation across loci was found to be 0.065 on the local region-level, and 0.114 among ponds within a region. Despite the higher absolute value of this differentiation index on the pond level, permutation tests revealed that allelic differentiation was higher than expected by chance among local regions (P=0.015), but not among ponds within a region (P=0.907). The region-level F_{ST} was calculated to be 0.010, while the pond-level F_{ST} was 0.003; corresponding tests also indicated that differentiation was significantly higher than expected by chance only at the regional level (region-level G*=170.65, P=0.019; pond-level G*=536.01, P=0.326). In pairwise comparisons, we found that only the 'Upper' (U) and 'Lower' (L) local regions differed significantly in terms of allelic differentiation, whereas there was only a weak tendency in difference between the 'Middle' (M) and 'Lower' local regions in this measure (U-L: ΔD= 0.069, P=0.011; U-M: ΔD= 0.077, P=0.307, M-L: ΔD= 0.084, P=0.073; Fig. 2). Similarly, region-level G* indicated that the 'Upper and 'Lower' local regions were significantly different in terms of nearness to fixation as well, while difference between the 'Middle' and 'Lower' regions was marginally non-significant (U-L: G*=100.24, P=0.009; U-M: G*=66.33, P=0.463; M-L: G*=74.48, P=0.070). In accordance with the above tests, AMOVA showed that local regions were genetically differentiated from each other (Φ=0.015, Sum of Squares=6.717, $d.f.$=2, P=0.018), with 1.5 % of genetic variation distributed among regions, while variation among ponds within regions was essentially zero (Φ=-0.010, -0.96 %; Sum of Squares=15.323, $d.f.$=7, P=0.824). MCMC simulations consistently retrieved two genetically different populations at two clusters of ponds (Fig. 3), indicating the presence of genetic differentiation between the 'Upper' and 'Lower' local regions that was also found in the permutation tests. We found no correlation between genetic and geographic distances between ponds (Mantel's test, r= -0.001, P=0.484; Fig. 4).

Table 1. Decomposition of genetic diversity in the study area. D_{γ}^{total} indicates gamma diversity on the ecosystem level, while D_{α} denotes alpha diversity and D_{β} beta diversity, respectively. Upper-case numbers indicate the hierarchical level at which given components were estimated (1: pond-level, 2: local region-level). ΔD denotes normalised differentiation indices. Values within the parentheses are one-tailed conservative P-values; significant differences are shown in bold.

Loci	D_{γ}^{total}	$D_{\alpha}^{(2)}$	$D_{\alpha}^{(1)}$	$D_{\beta}^{(2)}$	$D_{\beta}^{(1)}$	$\Delta D^{(2)}$	$\Delta D^{(1)}$
L1	18.414	15.987	11.175	1.152	1.431	0.136 (0.465)	0.289 (0.852)
L2	3.936	3.806	3.602	1.034	1.057	0.032 (0.099)	0.045 (0.778)
L3	4.793	4.524	4.101	1.060	1.103	0.056 (0.045)	0.079 (0.549)
L4	3.266	3.167	2.892	1.031	1.095	0.030 (0.638)	0.074 (0.289)
L5	5.139	4.717	4.160	1.090	1.134	0.083 (0.015)	0.101 (0.785)
L6	5.258	4.974	4.426	1.057	1.124	0.053 (0.159)	0.095 (0.665)

DISCUSSION

We examined the extent of genetic differentiation on the pond and local region levels between demes of smooth newts over a scale of about 3.5 km in a natural breeding habitat. We found that genetic differentiation on the local region level was significantly higher than what we would expect by chance, and this result was supported by the calculation of two differentiation measures and by the analysis of microsatellite variance. Pairwise comparisons of local regions suggested that this region-level differentiation was due to difference in both the actual allelic composition and estimated nearness to fixation (measured by hierarchical F_{ST} and Φ) between the

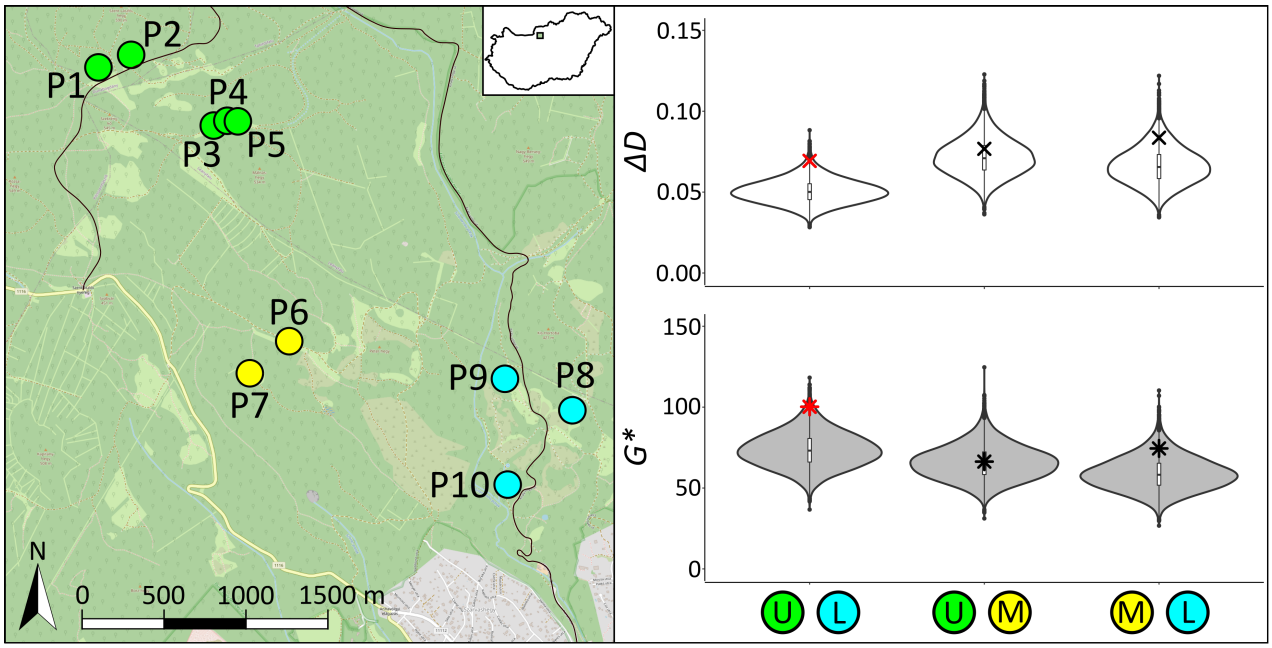

Figure 2. Locations of the breeding ponds (left panel) and null distributions of regional *ΔD* (normalised allelic differentiation index) and *G** (multilocus G-statistics) with the observed values for each pair of local regions (right panel). Ponds marked with different colours belong to different local regions (U: 'Upper' region, M: 'Middle' region, L: 'Lower' region). Violin plots indicate the distribution of differentiation indices (white: *ΔD*, grey: *G**) calculated from reshuffled datasets reduced to the two regions in question. Symbols (×: *ΔD*, *: *G**) denote the observed values calculated from the original data. Symbols in red indicate significant deviations from the corresponding permutation distributions. Between-region pond distances are shown in Table S1 and Figure S1. The map was created using OpenStreetMap (OpenStreetMap contributors 2015).

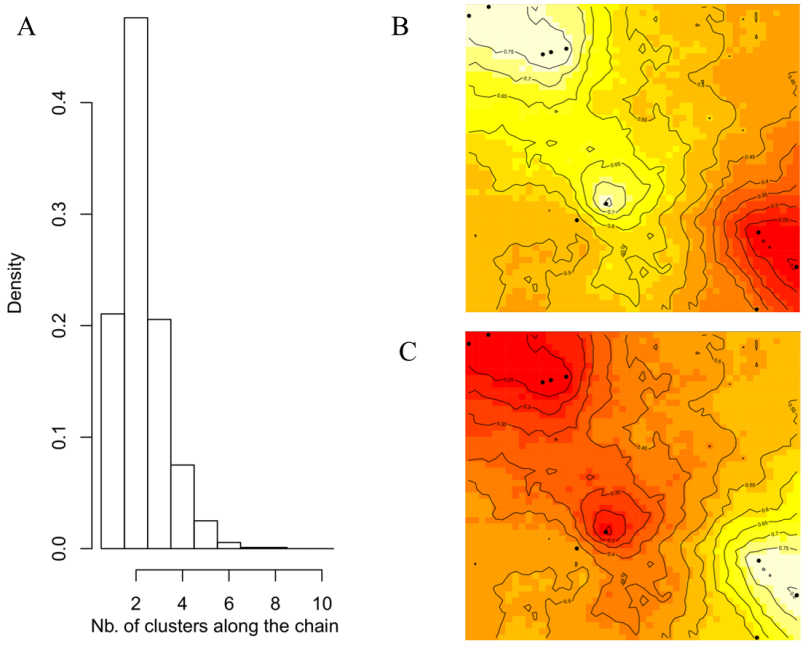

Figure 3. Bayesian clustering analysis conducted in GENELAND. **A)** Distribution of posterior probability of a number of genetic clusters (K). **B, C)** Maps of population membership probabilities for 107 smooth newts belonging to one of the two inferred clusters.

two furthermost regions. Genetic distance, however, did not correlate with geographic distance between ponds, indicating that isolation by distance was not likely to be the major cause of the detected genetic differentiation among local regions.

Our results support the previous findings of Schmidt et al. (2006) regarding low genetic variation in smooth newts on the studied spatial scale, and confirm that dispersal distance between demes may be far greater than implied by earlier studies (50-182 metres; e.g. Griffiths, 1984; Bell, 1977; Dolmen, 1981). As we found no significant genetic differentiation between the

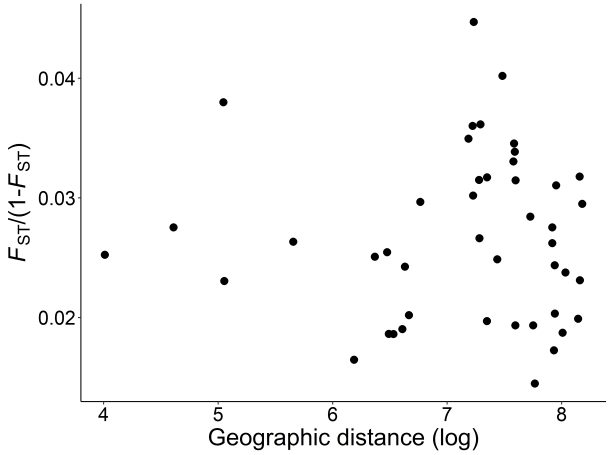

Figure 4. Relationship between genetic differentiation expressed as transformed F_{ST} and logarithm of geographic distances for the ten breeding ponds of smooth newts in the study area.

'Upper' and 'Middle' regions (this was also confirmed by the performed Bayesian clustering analysis), individuals were likely to be able to move among suitable breeding sites that are ca. 1.4 – 2 km apart under suitable weather conditions (e.g. on rainy days, as suggested by Weddeling et al., 2004). On the other hand, distances of 2.3-3.6 km between ponds in the 'Upper' and 'Lower' regions generated detectable differences between demes in the study area. Still, the estimated value of region-wise F_{ST} (0.010) was only a fraction of what is usually considered biologically significant because of reduced gene flow between populations (Frankham et al., 2010). Allelic differentiation measures also indicated that the number of local region equivalents was close to unity for most loci, and only 1.5 % genetic variation was distributed among regions. Our findings thus support and provide further genetic evidence for the idea that dispersal distance is likely to be underestimated in smooth newts and, in fact, individuals may regularly disperse between ponds, with some animals covering great distances and connecting apparently separate breeding populations (as suggested for many amphibians; Marsh & Trenham, 2001; Smith & Green, 2005). Although differentiation in fixation measures was found to be higher in some *Lissotriton* species than the estimated value of regional F_{ST} in this study, these discrepancies were rather related to the spatial scales on which those studies were conducted. For instance, in *L. graecus*, Sotiropoulos et al. (2013) showed that the overall genetic differentiation among demes at 10 breeding ponds in a semi-natural landscape was moderate (F_{ST} with 95 % CI: 0.039 [0.011, 0.092]), with geographical distance between localities ranging between c.a. 0.05 and 6.3 km. In the *L. vulgaris meridionalis*, Buono et al. (2017) showed that pairwise F_{ST} values between three locations that were 10.1–15.7 km far from each other ranged between 0.081 and 0.132. Previous works have pointed out that allelic and fixation differentiation measures do not provide the same information about the genetic variation in populations, but quantify complementary aspects of the prevailing genetic structure (Bird et al., 2011; Caballero & García-Dorado, 2013; Jost et al., 2018). Here we used both hierarchical F_{ST} and Shannon entropy-based *ΔD* indices for estimating genetic differentiation. Because the results for the two types of measures were in agreement, we are confident that the detected small, but significant local region-level genetic differentiation reflects a genuine pattern of genetic variation between smooth newt breeding ponds across the study area. Our results highlight that limited dispersal distance in amphibians should not be assumed automatically, but needs to be measured directly/inferred to indirectly from genetic data in the species of interest. The capacity of a species to exchange individuals between (sub-)populations is often determined by an interaction between spatial distance and individual land use. Some habitat features can facilitate animals' movement through a landscape. For example, water bodies that are unsuitable for reproduction still may serve as stepping stones connecting distant breeding sites, while habitat features representing barriers cause genetic discontinuities. In line with that, in the sympatric crested newts (*Triturus cristatus*), landscape factors such as forest gravel roads and south/south-west facing slopes contributed substantially to genetic differentiation between populations together with geographic distance (Haugen et al., 2020). In the palmate newt, *L. helveticus*, car traffic on secondary roads was found to select for short-legged newts due to a higher mortality of more mobile individuals characterised by long hind limbs, thus hind limb length, and consequently animals' dispersal ability, changed according to a landscape characteristic in the studied subpopulations (Trochet et al., 2016). In our study, both the permutation tests and the clustering analysis indicated that genetic distinction between two local regions, although roads did not separate them specifically. This finding, together with the lack of correlation between the observed distribution of genetic variation and geographic distance between ponds, implies that natural landscape parameters that were not taken into account here may have important roles in shaping genetic differentiation patterns in smooth newts as well. Previous studies have successfully integrated the identification of influential landscape characteristics into their investigations of connectivity between populations in various amphibian species (e.g., Atlas & Fu, 2019; Lenhardt et al., 2017; Haugen et al., 2020). We propose that a similar approach should also be adopted by future population genetics studies on this caudate species, as both natural barriers and human-induced environmental changes may affect first and most severely those occasional migrants that connect distant breeding populations, ultimately leading to hindered gene flow and strong genetic structure at relatively small geographical scales.

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experimentation in Hungary and the European Union, and was approved by the institutional ethics committee (Centre for Agricultural Research, Plant Protection Institute, Institutional Animal Care and Use Committee; ATK NÖVI MÁB) in accordance with Good Scientific Practice guidelines and national legislation. All sampling procedures and experimental manipulations of this study were reviewed and specifically approved by the national authority of the Middle-Danube-Valley Inspectorate for Environmental Protection, Nature Conservation and Water Management, who issued the permission to capture, handle and collect samples from the animals (KTF: 603-3/2014, KTF: 603-4/2014, KTF: 2771-3/2015). Map data copyrighted OpenStreetMap contributors and available from https://www.openstreetmap.org.

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