



Urban environment determines population genetics in the green toad, *Bufo viridis*

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Abstract

Heavily urbanized areas can hinder dispersal and gene flow between amphibian populations. Given the growth potential of urbanization, it is important to examine how this specific environment shapes their genetic patterns at the local scale. The ability of the European green toad to successfully colonize large human settlements has been convincingly confirmed in the recent past, but little is known about its population genetics under these new conditions. In this study, we examined the effects of the environment on genetic variation, population structure, and the level of gene flow in populations of this amphibian in the city of Košice and the adjacent rural area (eastern Slovakia) using a set of neutral genetic markers. We found that urban populations had lower genetic variability than populations in adjacent rural areas; however, the degree of inbreeding was relatively low in all samples. Genetic differentiation was higher, and gene flow was more restricted in urban area, although geographic distances between sites were significantly less than in rural area (2–4 km versus 6–13 km). Our analyses suggested genetic isolation of urban populations at sites with less suitable habitat for green toads. In contrast, admixture of the population inhabiting the large city park, established on a former floodplain, with all rural populations was likely the result of an intense historical gene flow. The densely developed environment of the other urban sites likely presents a strong barrier to gene flow. The lack of suitable wetland habitat prior to development suggests that these sites were only recently colonized by a limited number of founders. Thus, we found differential effects of the city on the population structure of the green toad. Understanding current local genetic variation and structure is important for future conservation plans in urban environments.

Keywords Amphibia · City environment · Dispersal · Founder effect · Landscape genetics · Microsatellites

Introduction

Since the 1950s, urbanization has become one of the most important processes altering natural landscapes worldwide, associated with degradation, fragmentation, and loss of habitats, as well as declines in many wildlife populations (Hamer and McDonnell 2008; Faeth et al. 2011; Elmqvist et al. 2013). At the global scale, an apparent decline in amphibians with aquatic larval development has been documented, significantly impacted by human-induced physical barriers between breeding pools and terrestrial habitats of adults (Stuart et al. 2004; Becker et al. 2007). Unidirectional

movement from the natal pond to new breeding sites is important for recolonization or persistence of metapopulations, especially in fragmented urban landscapes (Semlitsch 2008). Therefore, maintaining viable populations requires sufficient quality of both habitat types and connectivity between local populations (Trenham and Shaffer 2005; Harper et al. 2008).

Depending on the life history traits, mobility, and habitat preferences of amphibians, the landscape surface may provide resistance to dispersal and thus gene flow (Garcia et al. 2017; Cayuela et al. 2020). For example, forested areas resulted in the lowest resistance surface to gene flow for populations of long-toed salamander (*Ambystoma macrodactylum*), whereas it had the opposite effect for Columbia spotted frogs (*Rana luteiventris*). However, for both species, the highest landscape resistance resulted from urban and highly modified rural land areas (Goldberg and Waits 2010). Several studies have identified anthropogenic landscape features as barriers that reduce gene flow among amphibian

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populations and cause their isolation, which can lead to inbreeding, higher genetic differentiation, and increased genetic drift (e.g., Hitchings and Beebe 1998; Safner et al. 2011; Lourenço et al. 2017). Roads, fences, and buildings have been considered the most typical dispersal barriers in the urban matrix (Hamer and McDonnell 2008). In addition to the hindering effect of roads as impermeable surfaces which impact population connectivity, they can also reduce population sizes and local densities of anurans due to higher mortality following collisions with cars (Fahrig et al. 1995; Holderegger and Di Giulio 2010).

In highly urbanized areas, the viability of fragmented populations may benefit from habitat corridors that promote gene flow. For example, for populations of the natterjack toads (*Epidalea calamita*) living in urbanized coastal areas, dunes and beaches serve such a function (Cox et al. 2017). On the other hand, some pond-breeding species with higher dispersal abilities or larger home-ranges can maintain viable populations in urban areas (Richardson 2012; Furman et al. 2016). Thus, the same landscape features can have different effects on the population genetics of even co-occurring species (Brede and Beebe 2004; Homola et al. 2019). In addition to the average ability of species to move, occasional vagrants in particular, which can travel further distances of several kilometres, can shape patterns of gene flow between amphibian populations and thus play an important role in overall population survival (Smith and Green 2005; Ronce 2007; Sinsch et al. 2012). Therefore, anthropogenic landscape features may determine genetic structure in different ways, depending on the urbanization potential of the species and the specific circumstances of the urban area (Miles et al. 2019; Schmidt and Garroway 2021).

The European green toad (*Bufo viridis*) is an amphibian species that has successfully colonized human settlements in the recent past. Despite its limited movement ability (~800 m, Sinsch and Leskovar 2011) and small home range size (~0.25 ha, Indermaur et al. 2009), it is capable of colonizing diverse habitats (Degani et al. 2013) and it is a persistent urban dweller even in large European cities, for example, Rome (Ensabella et al. 2003), Kraków (Budzik et al. 2013), Cologne (Vences et al. 2019), or Warsaw (Mazgajska and Mazgajski 2020). However, there is limited information on how the urban environment shapes the genetic patterns of local populations (Vences et al. 2019). In the present study, we aimed to investigate the effects of different landscapes on the genetic patterns of populations of this species using a set of neutral genetic markers. We selected two contrasting landscapes, characterised either by native habitats or by urban structures to better understand population-genetics limits of this species to colonize novel environment. We examined genetic variation, population genetic structure, the level of gene flow, and signatures of demographic bottleneck in two groups of neighbouring

populations living in the city of Košice and in rural areas of eastern Slovakia, respectively. Since the species is generally considered to be a successful urban colonizer, we hypothesized that genetic variation and the level of gene flow between nearby populations would be similar regardless of the environment. However, an urban environment is highly site-specific in terms of habitat proportions and spatial structure, so we alternatively hypothesized that isolated urban populations will demonstrate signatures of demographic bottleneck and will have reduced variation and stronger genetic structuring due to restricted gene flow.

Material and methods

Study populations

The green toad is widespread in the lowlands and submontane regions of eastern Slovakia. There, six local populations were selected from two contrasting landscapes (Fig. 1). Three urban sites (Mestský park, ‘city park’—uMSP, 48.724° N, 21.264° E, 205 m a.s.l.; Sídliisko KVP—uKVP, 48.715° N, 21.212° E, 298 m a.s.l.; Zuzkin park—uZUZ, 48.719° N, 21.238° E, 243 m a.s.l.) were located in the agglomeration of Košice, the second largest city in Slovakia (ca. 240 km²; ca. 239,000 inhabitants). These sites were parks or recreational areas with varying amounts of tree vegetation (approximate size of this habitat: uMSP—13 ha, uKVP—0.8 ha, uZUZ—2.2 ha) separated by settlements of buildings, roads, and other human constructions. Artificial ponds—fountains with periodic or permanent water serving as breeding pools—occurred at each urban site. Geographic distance between urban populations ranged from 2 to 4 km. Three rural sites were located 15–23 km SW from the periphery of the city. Two of them (Cestice—rCES,

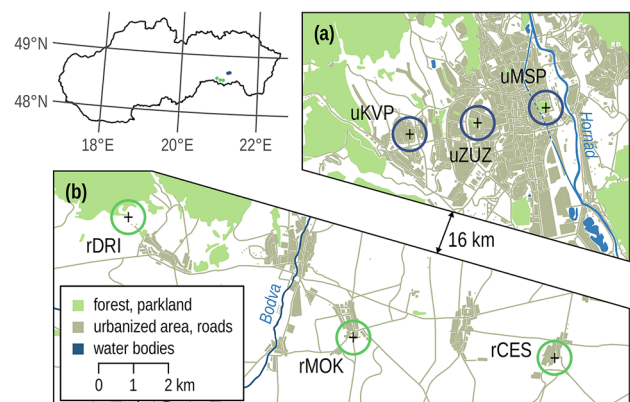


Fig. 1 Location of six study populations of *Bufo viridis* in eastern Slovakia (a, populations in the city of Košice; b, populations in the adjacent rural area) with landscape structures representing potential barriers to gene flow

48.589° N, 21.100° E, 203 m a.s.l.; Mokrance—rMOK, 48.592° N, 21.022° E, 194 m a.s.l.) were located in farmland with various shallow periodic pools, while one rural site (Drienovec—rDRI, 48.620° N, 20.932° E, 192 m a.s.l.) represented a limestone quarry with an artificial lake in the middle of cultivated fields. In contrast to the urban area, the average geographic distance between rural populations was much greater, ranging from 6 to 13 km. Twenty adults of *B. viridis* were sampled at each site during 2018–2022.

Genotyping

In total, we successfully genotyped 120 individuals using 11 previously described microsatellite loci (Vences et al. 2019). A tissue sample was obtained by toe clipping (the last phalange of the third digit of the right forelimb) and stored in 96% ethanol until analysis. Total genomic DNA was extracted with GeneJET Genomic DNA Purification Kit (Thermo Fisher Scientific). Microsatellite markers were amplified by three multiplex PCR protocols (Table 1) using Qiagen Multiplex PCR kit (Qiagen). Each PCR protocol required 1 µl of genomic DNA (~ 100 ng/µl), forward and reverse primers of each locus (molar concentrations in Table 1), a 1 × Qiagen Master Mix and a 0.5 × Q-solution in a 12 µl reaction volume. A thermocycler program for the PCR amplification involved an initial cycle of denaturation at 95 °C for 5 min, 35 cycles of denaturation at 95 °C for 30 s, annealing at 60 °C for 45 s, extension at 72 °C for 90 s, and a final elongation step at 72 °C for 5 min. The fluorescent-labelled PCR products (Table 1) were separated by capillary electrophoresis in an ABI 3730XL genetic analyser, and fragment lengths estimated relative to the size standard LIZ600 were

determined using Geneious Prime 2019 software (Biomatters). Scoring of peaks was performed by a single observer.

Microsatellite loci were checked for the Hardy–Weinberg equilibrium and frequency of null alleles using the package ‘PopGenReport’ 3.0.4 (Adamack and Gruber 2014) of the R 4.1.2 software (R Core Team 2021). In all populations but uMSP, 1–3 different loci deviated from the Hardy–Weinberg equilibrium ($p < 0.05$). Similarly, some linkage disequilibrium occurred in all populations. Because the linkage values differed from each other and no consistent pattern between any two loci was observed, loci were thus considered to be unlinked in the genome (see Vences et al. 2019). Loci exhibited in general low frequency of null alleles (up to 8% in Bvir3022) across all locations when tested using the Chakraborty et al.’s (1994) and Brookfield’s (1996) methods.

Genetic variation and differentiation

Basic characteristics of genetic variation in sampled populations i.e., the number of alleles (NA), observed (H_O) and expected (H_E) heterozygosity, and inbreeding coefficient (F_{IS}) were calculated using the R package ‘adegenet’ 2.1.3 (Jombart 2008). Differences among groups were determined by the non-parametric Kruskal–Wallis ANOVA with post-hoc Dunn test for multiple comparisons in the R package ‘FSA’ 0.8.30 (Ogle et al. 2020). An individual- and a population-based pairwise estimates of genetic distances among sampled populations were calculated using D_{PS} (Bowcock et al. 1994) and G'_{ST} (Hedrick 2005), respectively, in the R package ‘graph4lg’ 1.2.0 (Savary et al. 2020).

Table 1 Characteristics of 11 microsatellite loci (Vences et al. 2019) used in the present study

Locus	c (µM)	Dye	NA	H_O	H_E	F_{IS}
<i>multiplex 1</i>						
Bvir15979	0.1	ATTO 565	22	0.842	0.890	0.054
Bvir23976	0.2	6-FAM	16	0.908	0.900	−0.010
Bvir19382	0.3	Yakima Yellow	19	0.842	0.892	0.057
Bvir11064	0.1	ATTO 550	12	0.924	0.835	−0.107
<i>multiplex 2</i>						
Bvir10561	0.1	ATTO 565	14	0.892	0.819	−0.089
Bvir5761	0.2	6-FAM	17	0.975	0.847	−0.151
Bvir3372	0.4	Yakima Yellow	21	0.792	0.893	0.113
<i>multiplex 3</i>						
Bvir3022	0.1	ATTO 565	19	0.758	0.908	0.165
Bvir16861	0.3	6-FAM	15	0.825	0.891	0.075
Bvir29737	0.3	Yakima Yellow	22	0.942	0.909	−0.036
Bvir13782	0.1	ATTO 550	18	0.875	0.913	0.041

c molar concentration of both primers in multiplex PCR, dye fluorescent dye of forward primers, NA number of alleles, H_O observed heterozygosity, H_E expected heterozygosity, F_{IS} inbreeding coefficient

Bottleneck analysis

For testing demographic bottleneck, we defined the null hypothesis as being presence of mutation-drift equilibrium under a two-phased mutation model which combines the stepwise mutation model and the infinite allele model. First, we tested for a recent reduction in effective size, thus an excess in heterozygosity. In addition, we controlled for signatures of a bottleneck by the deficiency in M-ratio as the total number of alleles (k) divided by overall range in allele size (R) (Garza and Williamson 2001). Supposing that k decreases faster than R when the population size is reduced, M-ratio is expected to be smaller in recently reduced populations compared to populations in mutation-drift equilibrium. Thus, the heterozygosity test assumes that a bottleneck eliminates rare alleles, whereas the M-ratio test is based on the idea that drift removes alleles randomly with respect to allele size (Williamson-Natesan 2005). According to suggestion by Peery et al. (2012), proportion of multi-step mutations (p_g) was set to 0.22, and average multi-step mutation size (δ_g) was 3.1 during 100,000 coalescent simulations. Using the software INEST 2.2 (Chybicki 2017), both the excess in heterozygosity and the deficiency in M-ratio in each population were tested by the Wilcoxon signed-rank test which does not assume normality of data distribution and approximates the exact p -value after 1,000,000 permutations.

Population structure and gene flow

To identify genetically homogeneous populations of individuals in our samples, we applied two different approaches. Firstly, we examined this by a Principal Coordinates Analysis (PCoA) of the R package ‘adegenet’ 2.1.3 (Jombart 2008). This multivariate method considers the best approximation of a dissimilarity matrix of Euclidean and short distances while its principal components optimize the representation of the squared pairwise distances between individuals. Secondly, we used an individual-based clustering method implemented in the software Structure 2.3.4 (Pritchard et al. 2000; Hubisz et al. 2009) to infer optimal number of genetic clusters (K). We ran the admixture model with correlated allele frequencies without the prior population information and degree of admixture $\alpha = 1$. For each value of K (range 1–6), we conducted 10 independent runs with uniform priors using a burn-in of 100,000 iterations followed by 100,000 Markov chain Monte Carlo iterations. The optimal number of clusters in the data set was inferred by the ΔK method (Evanno et al. 2005), which finds the breakpoint in the slope of the likelihood distribution for different K values, using the Structure Harvester Web 0.6.94 (Earl and vonHoldt 2012) and also through Q-matrix correlations, which determine the stable K solutions (identify anomalous runs) implemented in the R package ‘CorrSieve’ 1.6–8 (Campana et al. 2011).

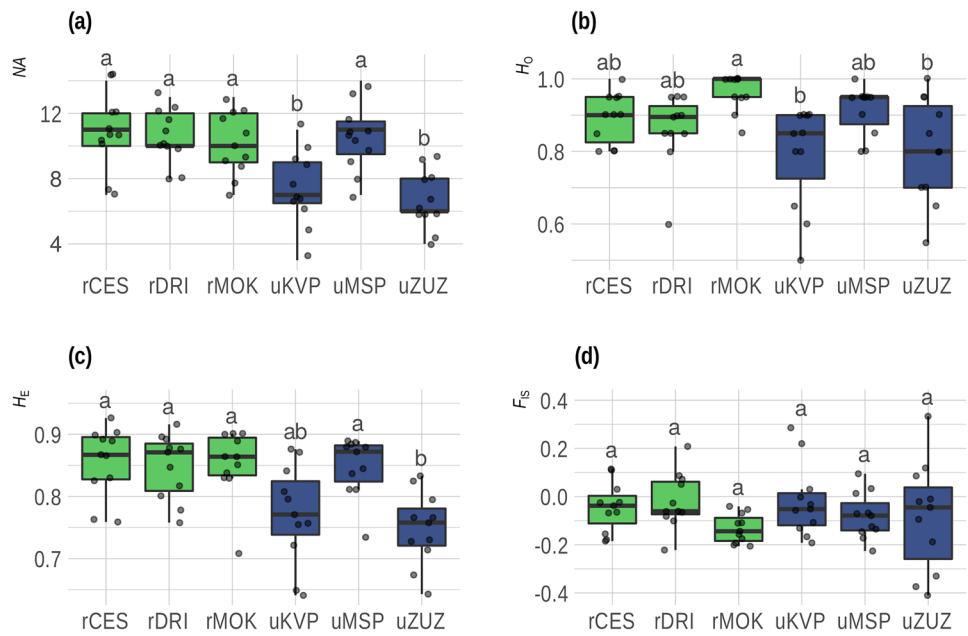
We assumed that individual migration between two geographically distant population groups would extend far beyond the dispersal limits of an individual of the species under study and that linkage of groups would overestimate possible evidence of gene flow within groups. Therefore, to examine gene flow, we computed directional relative migration values between populations in urban and rural areas separately using the divMigrate function (Sundqvist et al. 2016) from the R package ‘diveRsity’ 1.9.89 (Keenan et al. 2013). Statistical significance of directional migration was calculated using 1,000 bootstraps. Furthermore, we detected possible immigrants from neighbouring populations using a machine-learning framework implemented in the R package ‘assignPOP’ 1.2.0 (Chen et al. 2021). A naïve Bayes classification method (a simple probabilistic classifier with strong independence assumptions between features) was used as the default setting of this package to build a predictive model that estimated individual membership probabilities for each study population using baseline data representing all microsatellite genotypes of three populations of the corresponding area (rural or urban).

Results

All 11 microsatellite loci were highly variable, with 12–22 alleles per locus, and showed relatively high observed (0.758–0.975) and expected heterozygosity (0.819–0.913). The inbreeding coefficient ranged from -0.151 to 0.165 (Table 1). Genetic variation between populations differed significantly with respect to the number of alleles ($\chi^2 = 27.5$, $p < 0.001$) and heterozygosity (H_O , $\chi^2 = 20.9$, $p < 0.001$; H_E , $\chi^2 = 23.5$, $p < 0.001$). It was mainly decreased in two urban populations, uKVP and uZUZ. In contrast, uMSP urban population did not differ from rural populations (Fig. 2). Unique (private) alleles were found in all populations. The highest number of them was found in uMSP (14), and the lowest in uZUZ (3). Other populations displayed 5–7 private alleles. There were no significant differences in the F_{IS} index of the populations. Genetic differentiation of urban populations was higher (mean \pm SD; $D_{PS} = 0.608 \pm 0.049$, $G'_{ST} = 0.508 \pm 0.095$) compared to rural samples ($D_{PS} = 0.467 \pm 0.019$, $G'_{ST} = 0.247 \pm 0.055$), and this differentiation did not correlate with geographical distance between all sites (Mantel test with 999 replicates, $p = 0.72$). There were significant signatures of a demographic bottleneck ($p < 0.05$), which manifested as a deficiency in M-ratio in all populations, regardless of environment. However, we found no excess of heterozygosity (Table 2).

Multivariate PCoA analysis revealed significant variation in genotypes among individuals. The first two axes explained most of the variation (62.7%; Fig. 3a). Two urban populations (uKVP, uZUZ) differed significantly from the others, in contrast to uMSP, which did not differ from the rural sites.

Fig. 2 **a** Number of alleles, **b** observed heterozygosity, **c** expected heterozygosity, and **d** inbreeding coefficient in three rural (green) and three urban (blue) populations of *Bufo viridis* calculated from 11 microsatellite loci. Different lowercase letters above box-plots denote groups that significantly differ ($p < 0.05$) from the others according to post-hoc Dunn tests



In the outputs of the admixture model simulated in the Structure analysis, both methods ΔK and Q-matrix correlations inferred stable genetic structure at $K = 3$ (Fig. 3b, Fig. S1 of Supplementary information). However, Q-matrix correlations using both the maximum average correlation and the rows-and-columns criteria ($R = 0.99$, $p < 0.05$) yielded a stable solution also at $K = 5$ (Fig. S2 of Supplementary information). This suggests that each urban site represented a single genetic population, while all rural sites likely belong to one homogeneous genetic cluster.

Relative migration values between rural and urban populations also differed when using directional migration concept. Higher level of gene flow was calculated in the rural area, while lower level of gene flow between urban populations was significantly ($p < 0.05$) directional from the more isolated uKVP and uZUZ sites to the uMSP site (Fig. 4a).

Table 2 Results of two bottleneck tests for the null hypothesis of mutation-drift equilibrium under the two-phased model of mutation in populations of *Bufo viridis*. Z scores of the Wilcoxon signed-rank test and p-values based on 1,000,000 permutations

Population	Excess in heterozygosity		Deficiency in M-ratio	
	Z	p	Z	p
<i>Rural</i>				
rCES	0.09	0.483	-2.04	0.016
rDRI	-0.44	0.682	-2.40	0.005
rMOK	1.42	0.087	-2.49	0.004
<i>Urban</i>				
uKVP	0.71	0.260	-2.85	0.001
uMSP	-1.07	0.861	-1.87	0.027
uZUZ	1.24	0.120	-2.40	0.005

However, a machine-learning approach did not support this pattern in such resolution. Apart from two single immigrants between rural set of populations, all other individuals were assigned to the sites where they were sampled (Fig. 4b). Nevertheless, both analyses suggest very restricted gene flow in the urban area.

Discussion

In this small-scale study, we found that urban populations of *B. viridis* exhibit lower genetic variability, higher genetic differentiation, and spatial structuring compared to sites in the adjacent rural landscape, although different bottleneck tests provided discrepant results for the dataset analysed (see Peery et al. 2012). Our results clearly indicate that gene flow was more restricted in the city of Košice agglomeration, although the geographic distances between sampled sites were much smaller (2–4 km versus 6–13 km). Genetic differentiation and isolation were the most pronounced in two populations that reproduced at sites with the least amount of suitable habitat (uKVP, uZUZ). Surprisingly, the third urban population in the large city park (uMSP) was genetically homogeneous with rural populations outside Košice. Thus, we found differential effects of the city environment on the population structure of the green toad, as distinct urban populations could be probably the result of a recent colonization event or fragmented remnants of a historical population that lived in the area before heavy urban development. Thus, despite emerging markers and high-throughput technologies in population genetics, we confirmed that microsatellites can still be a powerful tool to draw inferences about population

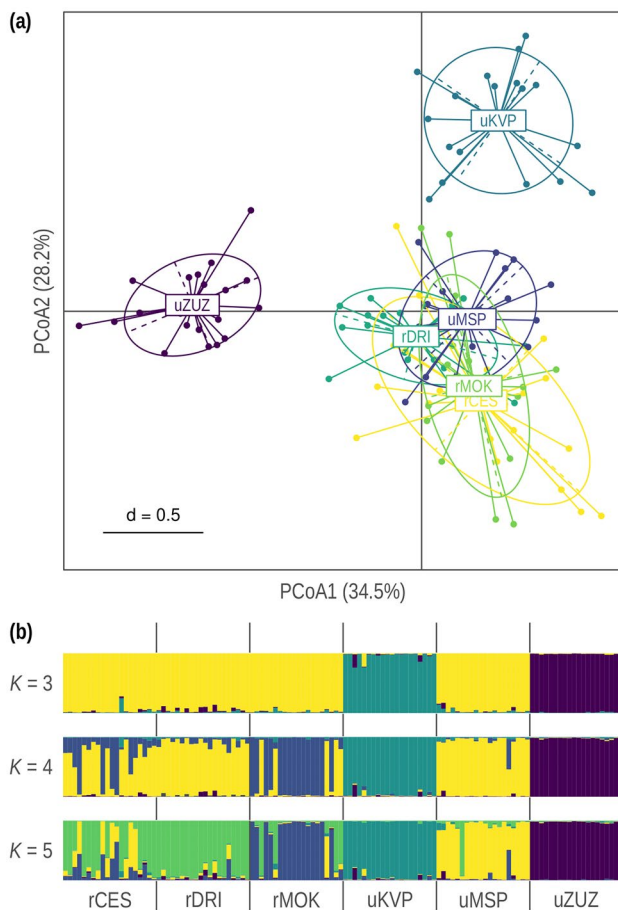


Fig. 3 **a** Genetic distances between individuals in the studied populations using the Principal Coordinate Analysis according to the first and second principal components with 95% inertia ellipse. **b** Genetic structure of samples in the study area inferred by the Structure admixture analysis. Individuals in the stacked bar plots of different K are represented by vertical bars divided into parts proportional to their proposed ancestry in determined genetic clusters

patterns and processes (Putman and Carbone 2014). To compensate for potential weaknesses of these markers, we have successfully integrated several complementary computational methods to answer our questions.

Urban populations are often founded by the immigration of individuals from the surrounding rural areas, while amphibians are generally considered species with weak dispersal abilities, often migrating only up to a few kilometres (e.g., Smith and Green 2005; Vences et al. 2019). In natural populations, they show a typical pattern of isolation-by-distance (Palo et al. 2003; Spear et al. 2005; Arioli et al. 2010) because *B. viridis* is a poor disperser (Indermaur et al. 2009; Sinsch and Leskovar 2011). However, greater geographic distance between populations does not necessarily correlate with greater genetic differentiation between them (Degani et al. 2013). This is likely due to small and isolated populations in urban environments, which should be more

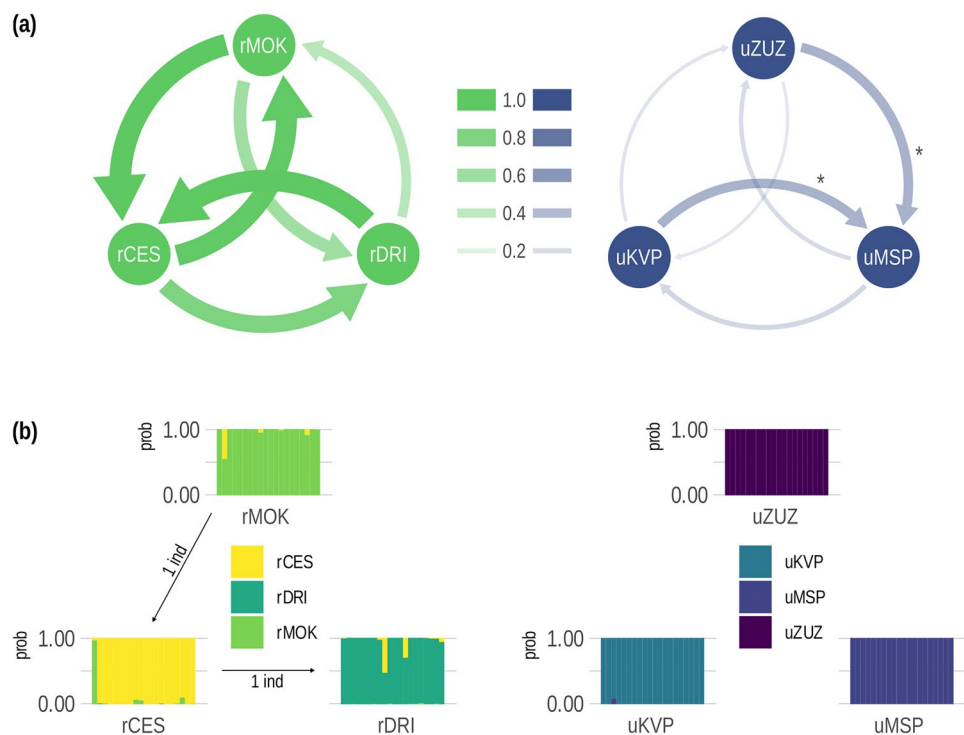
likely to exhibit genetic drift resulting from founder effect (demographic bottleneck).

Urbanization reduces the size of local populations and gene flow between them through habitat loss, degradation, or fragmentation (Johnson and Munshi-South 2017). In our urban populations, this effect was observed in lower genetic diversity and higher genetic differentiation compared to rural populations. Such a pattern was also found in populations of *Pelophylax ridibundus* by Mikulíček and Pišút (2012). In this case, very close (0.4 km) urban populations were genetically more differentiated ($F_{ST}=0.070$) than more distant populations (8 km) connected by a migration corridor of a water channel ($F_{ST}=0.010$), while neighbouring populations isolated by a four-lane highway were significantly more differentiated ($F_{ST}=0.062\text{--}0.089$). Similar results were demonstrated in *Rana temporaria*, although no physical barriers prevented the movement of individuals. Significant genetic differentiation ($F_{ST}=0.388$) was found between urban populations separated by 2 km compared to populations at less disturbed rural sites ($F_{ST}=0.145$) with an average distance of 41 km (Hitchings and Beebe 1997). In addition to the founder effect, strong selection pressure at breeding sites with unpredictable conditions (e.g., regular desiccation of pools due to temperature rise in the city) can produce populations with specific genotypes of adapted individuals, which can increase genetic differentiation among sites (Degani et al. 2013). Thus, the imbalance between migration and genetic drift may be a result of colonization of new sites by a limited number of founders or the creation of artificial barriers to gene flow (Mikulíček and Pišút 2012).

Both the Bayesian approach and multivariate analysis suggested genetic isolation of two urban populations at sites with less suitable habitat for green toads. In contrast, admixture of the population inhabiting the large city park with all rural populations and the highest number of private alleles is likely the result of intense historical gene flow (Smith and Green 2005). This site is located in the former floodplain of the Hornád River and its tributaries (Fig. 1), which could serve as a vector for migration of individuals from the surrounding area (e.g., Mikulíček and Pišút 2012; Vences et al. 2019). Thus, the city park was established on a natural wetland approximately 200 years ago (Municipality of Košice, personal communication) and still resembles the natural habitat of the species due to the high proportion of diverse vegetation, various roosting opportunities, and safe breeding pools in an extensive fountain system. In addition, the limited human presence (the park is closed to visitors at night) may accommodate toads in their nocturnal terrestrial activities (e.g., Rodríguez-Prieto and Fernández-Juricic 2005).

In contrast, the dense development around two other urban fountains in settlements on a terrace about 100 m above the level of the Hornád River, built in the 1960–1990s,

Fig. 4 Gene flow between rural (left) and urban (right) set of populations. **a** Calculated relative migration values between rural and urban populations of *Bufo viridis*. The larger of the two relative migration values indicates that it is likely to be a source population, and asterisk indicates a statistically significant ($p < 0.05$) directional migration. **b** Membership probability from the assignment test and the number of potential migrants (arrows). Individuals in the stacked bar plots are represented by vertical bars divided into parts proportional to their proposed membership to source population (coded in different colours)



is probably a strong barrier to gene flow. The lack of suitable wetlands prior to urban development suggests that these sites were colonized by a limited number of founders only after the construction of the fountains (strong founder effect). Based on the approximate generation time of the green toad (Sinsch et al. 2007), the effect of the demographic bottleneck there could already last 10–20 generations. If there is some gene flow between urban sites, it is more likely to be an occasional migration from small and regularly drained fountains towards the population in the city park below. To further examine this possibility, one should investigate the possible role of the city drainage system in the passive transport of individuals (cf. Semlitsch 2008). Another explanation for possible long-distance migration and the occurrence of private alleles in the city park could be anecdotal evidence of voluntary rescue transfers of some toads from other sites or even transport of goods, as it is located close to the main railway station.

Although the green toad successfully inhabits urban areas throughout Europe, its life history traits might differ due to specific local environmental conditions in cities, including intensity of development, pond water regime, predation, anthropogenic stressors, or chemical pollution (e.g., Ensabella et al. 2003; Kaczmarek et al. 2019; Mazgajski and Mazgajski 2020). Nevertheless, the low levels of the F_{IS} index in all populations in our study suggest that the species is not yet suffering from increased inbreeding even in the most isolated urban populations. Also, examination of the level of fluctuating asymmetry, which could serve

as an indicator of urban-induced environmental stress, revealed no significant differences between rural and urban populations (Vargová et al. 2022). However, considering the planned reconstructions of the fountains and their surroundings, the survival of the green toad may be at risk (Municipality of Košice, personal communication).

Future conservation plans for the studied populations should therefore include habitat protection and management (Semlitsch and Bodie 2003; Wei et al. 2021), especially maintaining adequate hydroperiod length in breeding ponds (Guderyahn et al. 2016), suitable roosting and wintering sites within sufficient distance from the breeding pond, and connectivity between sites (Furman et al. 2016). Green spaces, such as the large urban park in our study, typically function as species refugia in urban landscapes (Niemeier et al. 2020) and could be core habitats that provide sources for other populations. However, we believe that within the urban area of Košice, it is almost impossible to establish habitat connectivity that could maintain gene flow between small populations. Therefore, at least in the case of fountain reconstruction or temporary draining of the pool, maintaining green areas around the breeding site could benefit toad populations by providing roosting opportunities throughout the season (Semlitsch and Bodie 2003; Guderyahn et al. 2016; Wei et al. 2021). In terms of species adaptation to new and changing urban environments, long-term monitoring of local genetic variation and structure is therefore important for the favourable status of species in Central Europe (Sinsch et al. 2007).

Supplementary Information The online version contains supplementary material available at <https://doi.org/10.1007/s10344-023-01716-9>.

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Author contribution MU and MB conceived the idea, and PK designed the methodology; VV, MB, and NP collected samples; DG and VV performed laboratory work and genotyping; PK analysed the data and prepared figures; VV and PK drafted the manuscript; MU supervised the project. All authors discussed the results and edited the manuscript.

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Data availability The microsatellite data presented in this study are available on request from the first author.

Declarations

Compliance with ethical standards All applicable institutional and/or national guidelines for the care and use of animals were followed.

Conflict of interest The authors declare no competing interests.

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