

CZECH UNIVERSITY OF LIFE SCIENCES PRAGUE FACULTY OF
ENVIRONMENTAL SCIENCES (FES)



GENETIC STRUCTURE OF FIRE SALAMANDER (*Salamandra
salamandra*) IN URBAN ENVIRONMENT

DIPLOMA THESIS

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In Prague on

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ABSTRAKTNÍ

Mlok ohnivý (*Salamandra salamandra*) je veden jako „zranitelný“ v Českém červeném seznamu ohrožených druhů a jeho populace stále výrazně klesají v důsledku degradace a fragmentace jejich přirozených stanovišť způsobených zejména klimatickými změnami a urbanizací. Ačkoli různé studie zkoumaly populační genetiku mloka ohnivého v jeho přirozeném prostředí, je málo známo o genetické rozmanitosti a struktuře jejich populací v městských oblastech. V této studii bylo genotypováno 179 jedinců s 10 mikrosatelitovými lokusy sesbíranými z 5 různých míst, aby se odhadla genetická struktura a genetická diverzita mezi městskými a venkovskými biotopy. Současná studie nezjistila žádné významné rozdíly mezi městskou a venkovskou populací mloka ohnivého, což má za následek nízkou až střední úroveň genetické diverzity. Ukazuje, že tok genů byl na některých studovaných lokalitách omezený, což mohlo být způsobeno přítomností sídelních oblastí a řeky Vltavy. Tato studie uvádí, že obyvatelstvo, které leží v městských oblastech, mělo značný stupeň diferenciace od venkovských oblastí. Hlavním důvodem může být rozšiřování měst, silnice, dálnice, narušení vodních toků nebo potoků a méně zeleně v okolí městských oblastí. Nález severní populace (Pop 1) jako odlišné populace od zbytku však ukazuje adaptaci mloka ohnivého v severní oblasti Prahy. Nedávné rozšíření houby *Batrachochytrium salamandrivorans* (Bsal), stejně jako expanze měst, jsou vážnými obavami pro levou populaci mloka ohnivého v evropských regionech a vyžadují další výzkum, aby bylo možné vytvořit lepší plány ochrany. Podle aktuální studie však oblasti s roztržštěnými městskými populacemi mloka ohnivého vyžadují více výzkumu a městské správy, aby mohly poskytnout lepší útočiště.

Klíčová slova: městská genetika, ohniví mloci, mikrosatelity, populační genetika, *Salamandra Salamandra*.

ABSTRACT

The fire salamander (*Salamandra salamandra*) is listed as “vulnerable” in the Czech Red List of Threatened Species and their populations are still significantly declining due to degradation and fragmentation of their natural habitats caused mainly by climatic changes and urbanization. Although various studies investigated in population genetics of fire salamander in its natural environment little is known about the genetic diversity and structure of their populations in urban areas. In this study, 179 individuals were genotyped with 10 microsatellites loci's collected from 5 different sites to estimate genetic structure and genetic diversity between urban and rural habitats. The current study found no significant differences between urban and rural populations of fire salamanders resulting in a low to moderate level of genetic diversity. It shows that the gene flow was limited in some of the study sites which could be due to the presence of settlement areas and the Vltava River. This study reports that the populations which lie in the urban areas had a considerable degree of differentiation from the rural areas. The major reason behind it could be urban expansion, roads, highways, water or stream disturbance, and less greenery around the urban areas. However, the finding of the northern population (Pop 1) as a distinct population from the rest shows the adaption of fire salamander in the northern region of Prague. The recent spreading of the fungus *Batrachochytrium salamandrivorans* (Bsal), as well as the urban expansion, are some serious concerns for the left populations of fire salamander in the European regions and it require further research to make better conservation plans. However, according to the current study, areas with fragmented urban populations of fire salamanders require more research and urban management in order to provide better refuge.

Keywords: city genetics, fire salamanders, microsatellites, population genetics, *Salamandra Salamandra*.

1. INTRODUCTION

Amphibians are known as a good bio-indicator of environmental health; and salamanders with their higher position in the food chain, and complex life history, and sensitivity to environmental change, makes them an easy sample species to be studied as an indicator (Hermý and Cornelis 2000, Collins and Storfer 2003, Frisble and Wyman 1995, Welsh and Droege 2001, Venier et al. 2007, Bazar et al. 2008). Specifically, fire salamanders (*Salamandra salamandra*) populations, which is widely distributed in highly populated Europe, North Africa, and Middle East (Thorn 1986) are getting affected by potential problems such as climate change (seasonal and annual pattern of temperature and precipitation; Dervo et al. 2016), water acidity which leads to low availability of oxygen thus, leading to reduced growth of salamanders (Wyman and Hawksley-Lescault 1987), and light pollution (Disrupting photoperiod, Ecology, and Behaviour, Wise and Buchanan 2006). However, the sudden emergence of pathogenic infectious diseases such as the fungus *Batrachochytrium salamandrivorans* (Bsal) has brought fire salamanders to the brink of extinction within the short duration of approximately 5 years or less (Sluijs et al. 2018, USGS.gov).

Apart from this, the major factors are habitat destruction in the form of clearing of forest for timber and converting them into cultivated lands, expansion of urbanization, and wetlands drainage areas (Baillie et al., 2004, Stuart et al., 2004; Beebee and Griffiths, 2005; Cushman 2006, McKinney 2002, 2006). The gradual increase in urban areas tends to limit the environmental resources which lead to the isolation of populations, resulting in the elimination of species by reducing gene flow and loss of genetic diversity through genetic drift and inbreeding effects (Alvarez et al. 2015).

The effects of urbanization on population genetics have been studied primarily in highly mobile animals as those species are generally dependent on the resources provided by humans such as mammals (Rhesus monkey (*Macaca mulatta*), squirrels, birds (House sparrows (*Passer domesticus*), Common pigeons (*Columbia livia*), Fruit bats (*Peteropodidae spp.*), and many rodent species (e.g., Norway rat (*Rattus norvegicus*), Roof rat (*Rattus rattus*) which usually have higher density in urban habitat. But little attention is paid to animals with restricted mobility such as frogs and salamanders (Mason 2000, Castillo et al., 2003, Mckinney 2006, Møller 2009, Bókony et al. 2010, 2012, LaZerte et al. 2016, Johnson & Munshi-South 2017, Fidino et al. 2020). Many studies have been carried out on different salamander species showing uncertain results. While studies done on stream salamanders (Dusky salamanders)

which showed that urbanization had an adverse effect on its genetic connectivity and abundance (Price et al. 2006, Hamer & McDonnell 2008, Price et al. 2012, Munshi et al. 2013, Barrett & Price 2014, Fusco et al. 2020) others have observed moderate or no sign of genetic differentiation in fire salamander populations (Steinfartz et al. 2007, Straub et al. 2015, Yannic et al. 2021).

However, the main focus of this study is to understand fire salamander population genetics in urban environment and answer reasonable questions in order to make better conservation plans. Does the genetic structure and diversity of fire salamanders (*Salamandra salamandra*) populations in urban and non-urban areas differs? This study predicted that the individuals in the urban habitat would show greater genetic differentiation in comparison to the rural areas. Apart from this, I also predict that the genetic diversity and gene flow would be lower in the urban habitat than in the rural areas. The literature reviews in this study has provided important information's on city genetics and its influence on different groups of animals focusing on genetic diversity, structure and dispersal activity. As there had been very few studies specifically on fire salamanders, my major goal was to grasp a baseline for this study and specify management plans.

2. LITERATURE REVIEW

2.1. URBANIZATION CONSEQUENCES ON DIFFERENT SPECIES

The steady increase of urbanization over time is considered as one of the major factors which creating global pressures on the environment (UNHSP 2004, UNDP 2004, Angel et al. 2011, Seto et al. 2012, UN 2014, Knoema 2020, Grimm et al. 2008, Munshi et al. 2010). As more people live or migrate to the cities than the rural areas, it creates more demands for human settlements, resulting in the conversion of the natural landscape into buildings and roads (Marzluff 2001, Seto et al. 2012). Generally, urbanization is characterized by lower genetic diversity of species with scattered communities. Many species which are sensitive to environmental changes are facing predation, competition, or disturbance reflecting an adverse effect on the population decline. Whereas some of the species take advantage of this new characteristic of urban areas like lower predation risk, temperature, and changes in the food sources (Gering and Blair 1999, Marzluff 2001, Shochat 2004, Ditchkoff et al. 2006, Murphy et al. 2016). The effect of urban areas has been typically studied on the highly mobile species, but little attention has been given to the animals with reduced mobility (Tab 4). The dispersal ability of an organism plays a crucial role in predicting the gene flow and it also alters the genetic differentiation. Less mobile species such as amphibians and small mammals were expected with low genetic diversity in the area degraded by urbanization while urban organisms like Birds (House sparrows (*Passer domesticus*), common pigeons (*Columbia livia*)), squirrels, rhesus monkeys (*Macaca mulatta*), fruit bats (*Peteropodidae spp.*) are highly mobile and experience less decrease in genetic diversity with greater chances of survival in the city area than the outsiders (Møller 2009, Bókony et al. 2010, Munshi et al., 2013, Blanchong et al., 2013, Miles et al., 2019, LaZerte et al. 2016).

Among all vertebrates, amphibians have the highest proportion of species on the verge of extinction (41%), followed by mammals (25%), reptiles (22%), and birds (13%). (Stuart et al. 2004, Hoffmann et al. 2010, Pimm et al. 2014). Amphibians are known to be sensitive to environmental changes, and the effects of increased urbanization can result in decreased amphibian diversity (Effland and Pouyat 1997, Paul and Meyer 2001, Miltner et al. 2004, Carrasco et al. 2017). They possess highly permeable skin which is vulnerable to toxic substances, habitat loss, fragmentation, and urban stressors

such as noise pollution, light pollution, temperature, water pollution, and soil contamination (Mckinney 2002, 2006, Rubbo and Kiesecker 2005). However, the recent emergence of fungi such as *Batrachochytrium salamandrivorans* (Bsal) has proven to be a deadly barrier for amphibian populations, particularly fire salamanders (Stegen et al. 2017, Slujis et al. 2018). Amphibians provide many ecological functions to the environment, including significant biomass in forest and wetland ecosystems, as well as carnivore and prey species (Burton and Likens 1975, Gibbons et al. 2006). The fire salamander is one of the amphibian species threatened by extinction as a result of urbanization. Several studies in its natural habitat have been conducted, primarily on amphibians, but few studies have been conducted to determine the effect of urbanization on fire salamanders. However, studies on fire salamanders in city areas have shown low to moderate level of genetic diversity due to fragmentation effects but the actual reason behind it was uncertain and sceptical Munshi et al. 2013 (New York city); Fusco et al. 2020 (Metropolitan areas of New York); Alvarez et al. 2015, Lourenco et al. 2017 (Oviedo, Spain); Staub et al. 2015 (Salzburg, Austria). Apart from this, studies done on different species like the white-footed mice by Munshi-South and Kharchenko (2010), Lu et al. (2010), Munshi-South (2012), Munshi and Nagy (2014), Jonathan et al. (2020) in New York City, and metropolitan areas of USA shows the urban effects on species, as the white-footed mice (*P. leucopus*) population gets affected by the urban fragmentation leading to low genetic structure and suggested that forest with greater than 50 ha could support a large population of native small mammals.

Overall, increased urbanization has a negative impact on biodiversity in terms of habitat loss and fragmentation affecting the genetic diversity and structure of animal species, but the negative effects can be mitigated by creating parks or gardens in urban areas that preserve at least a portion of the original landscape (Noel et al. 2007). However, more research needs to be conducted to better understand the impact of urban expansion in genetic structure of animal species and to develop better conservation plans.

2.2 ANTHROPOGENIC BARRIERS AND THEIR EFFECT ON THE GENETIC POPULATIONS

The genetic variation in an area shows an ideal site which can be seen through the composition of a landscape and its features which are quite helpful for the gene flow. However, the genetic connectivity and the genetic drift are interlinked with each other. If there is a restriction in genetic connectivity it alters the genetic drift causing a decrease in genetic diversity, increase inbreeding potential, and decrease in reproductive success (Carlen and Munshi 2020). A brief review of the landscape and urban barriers could provide a better explanation of genetic level consequences on species. As habitat fragmentation associated with urbanization makes metapopulation structured species (amphibians, mammals) particularly vulnerable (Graham et al. 2017). These species are distributed across a heterogeneous landscape in discrete subpopulations, and they are reliant on dispersal among constituent subpopulations to some extent (Hanski 1998).

2.2.1 LANDSCAPE BARRIERS

The landscape genetics studies provide an approach, to measure and identify effective conservation and to maintain the functional connectivity among populations (Emel and Storfer 2015). For example, Anton et al. (2012) observed a high level of genetic differentiation and absence of gene flow of *S. salamandra* between the island and coastal population at Galician Atlantic Islands National Park. Seawater was considered an effective natural barrier to dispersal and gene flow for *S. salamandra* even for shorter distances (3.6-6km). Peterman et al. (2014) observed the landscape and ecological factors such as canopy cover, temperature, solar exposure and distance from ravine affecting the gene flow of terrestrial woodland salamander (*Plethodon albagula*) at East-Central Missouri. They concluded that the genetic diversity of woodland salamanders in major landscapes decreases as the water loss increases. However, the dispersal activity was also affected in the rainy season leading to a decrease in gene flow in favourable areas. Konowalik et al. (2016) in the Polish part of the Sudetes at Poland, observed genetic homogeneity of fire salamanders with no significant barriers (expansion of agriculture, road networks) to the gene flow; Beebee (2005) pointed to this study that a small or fragmented population can consist of stable genetic variation which could be the result of large effective population size. A recent

study by Yannic et al. (2021) in Central Switzerland observed moderate genetic differentiation of *S. salamandra* among sites with limited restriction to gene flow. Their landscape hypothesis (an assumption which shows that the landscape features have any positive or negative effect on the species) had mixed results representing minor effects of the barriers (large rivers, highways, and urban areas) on the genetic structure of the species. In contrast, Voros et al. (2017) found that the settlement areas of Budapest acted as a barrier to gene flow to the nearest salamander population in their study of fire salamanders in Hungary's Carpathian basin. Areas with no barriers and continuous forests, on the other hand, showed a high level of genetic differentiation. Blank et al. (2012) studied the northern Israeli population of *S. infraimmaculata*. They observed limited gene flow between Mount Carmel and Galilee region separated by low elevation and wide valley regions. The isolation at the mount Carmel region leads to low genetic diversity and possible chances of vanishing this species via strong genetic drift, bottleneck, and/or founder effect. Overall, most of the studies have observed mixed results depending on the site as well as anthropogenic effects leading to the genetic structure of the study species in their respective areas.

2.2.2 URBAN BARRIERS

Various studies had suggested the effect of fragmentation is caused by agriculture or logging activities on different species (Kolozsvary and Swihart 1999, Vos et al. 2001). However, urban development has also become a major factor of landscape changes and habitat fragmentation (Miller and Hobbs 2002). Mikulíček & Pišút (2012) at Bratislava city in Slovakia showed that marsh frog (*Pelophylax ridibundus*) populations had higher genetic connectivity within water path in the Danube river and artificial canals than populations from isolated ponds in the human-dominated lands. Straub et al. (2015) in Salzburg (Austria) expected the effect of anthropogenic barriers (urbanized areas, highways, and diked river) on the spatial genetic structure of Fire salamander. But there was no sign of overall genetic differentiation in fire salamander; suggesting that the population belongs to the one genetic pool. Similarly, Noel et al. (2007) studied on Eastern red-backed salamander (*P. cinereus*) in Montreal, Canada. Their comparison of the populations from fragmented and continuous landscapes showed a lower genetic diversity of the populations at mount royal park due to the effect of habitat fragmentation. Homola et al. (2019) worked on the spotted salamander

and wood frog in Maine, USA. They observed that the connectivity was restricted by roadways and rivers leading to poor gene flow of spotted salamander and wood frog. Carlen and Munshi (2020) at 6 metropolitan areas within North-eastern megacity studied the activity of feral pigeons. It shows high genetic connectivity, with little genetic differentiation between cities due to significant dispersal by some individuals across the urban landscape. Richardson et al. (2021) in Rhode Island metropolitan areas in the USA observed the difference in dispersal activities revealed the effect of urbanization, as mice leads to inbreeding whereas bats had weak genetic structuring but still they maintained a stable genetic diversity due to random mating in the study area. Alvarez et al. (2015), Grant and Lourenc et al. (2017) investigated a small, isolated population of fire salamanders trapped in Oviedo (Spain). They obtained a high ratio of N_e/N using the capture-mark-recapture method, implying that multiple paternity may contributed to the maintenance of variation and, to some extent, prevent inbreeding depression in small populations.

Schmidt and Garroway (2020; 2021), Browne et al. (2009), Coster et al. (2015), Furman et al. (2016), Lourenço et al. (2017), Nowakowski et al. (2018), Purrenhage et al. (2009), Wilk et al. (2020) in North America, observed the effect of urbanization on amphibians. They found species-specific responses of cope's giant salamander (*Dicamptodon copei*), rocky mountain tailed frog (*Ascaphus montanus*), and spring peeper (*Pseudacris crucifer*) with high genetic diversity in urban areas while species like Northern dusky salamander and California red-legged frog had shown a decline in the genetic diversity with the increase in urbanization.

2.3 DISPERSAL AND ADAPTATION OF AMPHIBIAN SPECIES

Dispersal is a crucial life history trait that has direct implications for gene flow, population dynamics, and persistence (Colbert et al. 2001). Dispersal may reduce local extinction rates by increasing genetic diversity in dispersed areas through a high reproduction rate (David et al. 2007). Amphibians have long been thought to have high breeding site fidelity and low dispersal rates (Blaustein et al., 1994). Recent research's shows that amphibians have a diverse range of dispersal strategies with some species capable of covering much greater distances than previously thought (Marsh and Trenham 2001, Trenham et al. 2001, Funk et al. 2005, Smith and Green 2005, Cushman 2006). Past studies has observed that an adult fire salamanders can move on

average 200-400 m with maximum direct distance of 1100-1300 m between the capture-recapture sites (David et al. 2007). However, increasing habitat fragmentation due to anthropogenic activities such as expansion of agriculture, urbanization, road network had adverse effect on the adaptation and dispersion rate on fire salamander species. As elevation is one of the factors which influence the habitat suitability of fire salamander. Wagner et al. (2020) study in the north-western part of the German Federal State of Rhineland-palatinate, shows that the larvae of *S. salamandra* abundance were negatively affected by elevation (Thiesmeier & Dalbeck 2011) and increasing agricultural land-use which led to herbicide contamination affect body size of larvae. Though, the high number of pools and shelters had a positive response to the abundance of the species. However, study by Jadczyk 2009, Mazurski 2014 described that deforestation (39,000 ha) in the boundary area of Polish-Czech Republic lead to the adverse effect on the invertebrates and vertebrates.

2.4 PROPOSED CONSERVATION MEASURES FOR ANIMAL SPECIES

In context to suggest and develop conservation measurements, Saarikivi et al. (2013) at southern Finland observed the genetic differentiation of common frog (*Rana temporaria*) between the golf courses and nearby natural sites. The low genetic differentiation across the study area shows that an adequate amount of greenery/habitat and water bodies were available to maintain a single large population. Munshi and Nagy (2014) on the white-footed mouse (*Peromyscus leucopus*) studied its genetic connectivity and diversity at NYC. Results show that >50 ha forest fragment can support a large population of native small mammals and eliminate the negative effect of genetic drift. Furman et al. (2016) in Alberta, Canada observed that the population of wood frogs (*Lithobates sylvaticus*) was affected by increasing urbanization, but the development of constructed wetlands has helped in the existence of wood frogs suggesting more development of constructed wetlands will preserve urban wood frogs and related species.

Fusco et al. (2020) study on northern two-lined salamander (*E. bislineata*) at New York City suggested that to minimize the effects of urban barriers, it is necessary to maintain urban greenspace to balance genetic connectivity of species. A general study by Fidino et al. (2020) on multi-species (mammals) in 10 cities of the USA. They observed the common species (Striped skunk, Red fox, Cottontail, Coyote, Gray

squirrel, Fox squirrel, Opossum, Raccoon) response to increasing urbanization with the help of camera traps. They observed that the response of red fox (*Vulpes vulpes*) and eastern gray squirrel (*Sciurus carolinensis*) within the city fluctuates if a city had less than 20% greenspace. They suggested that an average housing density higher than 710 housing units/km³ will affect the species at a local scale with increasing urbanization.

3. STUDY SPECIES

3.1 FIRE SALAMANDER AND ITS HABITAT PREFERENCES

Fire salamanders (*Salamandra salamandra*, Linnaeus, 1758), (Family= Salamandridae, 21 genera and 121 species) show a suitable example of biological polymorphism within a species and the major morphological variation includes in size (13-22 cm in snout-vent length for adult females), body proportions, head shape, and coloration (Buckley et al. 2007, Lourenco 2019). The body size of a female is larger as compared to the male and it also has larger interlimb distances (Labus et al. 2013). For differentiating between males and females, the external features like yellow spots on the dorsal surface and the presence of receptaculum seminis in the female's cloaca are commonly observed (Francis 1934, Opatrný 1983, Balogova and Uhrin 2015). However, in the breeding season, it is quite difficult to identify sexes due to the absence of secondary sexual characteristics in males (Balogova & Uhrin 2015).

The species prefer wet cool deciduous, mixed, or sometimes coniferous forests with shaded brooks and small rivers or running water. Being a good indicator, the species could also observe in woodless mountain pastures and hayfields, showing the existence of forest in the past IUCN (2009). Balogova et al. (2012) described that in Slovakia, the characteristics habitats for the salamander species are deciduous or mixed forest especially beech between the 200 and 1000 m of elevation. The mean home range of males was 511.3 m² (3.32 m² —3200 m²) and of females 151.3 m² (23.4 m²—443 m²). In another study by Balogova and Uhrin (2014) observed, significant movement of females as compared to the males at underground sites of eastern Slovakia. Many individuals change their position in winter shows the roosting site preferences as open or a half-hidden position and their occurrence is observed in caves and underground roosts Balogova et al. (2015).

3.2 REPRODUCTION STRATEGY

The biphasic life cycle of *S. salamandra* possesses two co-occurred reproductive strategies i.e., viviparity and pueriparity. In larviparity, the females give around 90 larvae in the streams and ponds after a gestation period of approximately 90 days while in pueriparity it delivers around 1-35 fully metamorphosed terrestrial juveniles within the same gestation period (Buckley et al. 2007, Greven 2003). The major

characteristics of viviparity in *S. salamandra* are the early hatching of the embryos which takes place in the maternal oviduct through the phenomena of oophagy and adelphoghagy (Wourms 1981, Joly 1986). Ovoviviparous females lay around 20 to 60 eggs which upon fertilization, development takes place within the uterus and hatches while hatching in ovoviviparous *S. salamandra* happens in the middle or before the dissemination of larvae into the water Buckley et al. (2007).

3.3 DISTRIBUTION AND THREAT STATUS

According to IUCN (2009), the species is distributed across Central, Eastern, and Southern Europe (Fig. 1). The species is widespread in Slovakia (Balogova et al. 2012) while in the Czech Republic, the species is critically endangered and protected by international legislation (Jeřábková et al. 2017, Brejcha et al. 2021, National Museum, CZ). Specifically, the Fire Salamander is one of the amphibian species which is considered as “Least Concern” by the IUCN Red list data (2009) while it is listed as “Vulnerable” by the Czech Red List of Threatened species (Jeřábková et al. 2017). In central Europe, two evolutionary lineages of *S. salamandra* had been observed and considered distinct subspecies. The banded fire salamander (*S. salamandra terrestris* Bonnaterre, 1789, Brejcha et al. 2021; Two parallel strips), typically scattered from Pyrenees to Germany whereas the spotted fire salamander (*S.s. Salamandra* Linnaeus 1758; Irregular isolated spots) distributed throughout eastern Germany, Central and Eastern Europe, and the Balkans (Thiesmeier & Grossenbacher 2004, Seidel and Gerhardt 2016) differentiated with each other by dorsal colour pattern. The current population trend of the species is decreasing because of urban and commercial development, agriculture, and aquaculture, use of biological resources, Invasive and other problematic species, and pollution IUCN (2009).



Fig. 1 Geographic range of *S. salamandra* by IUCN (2008)

4 STUDY AREA

The study was carried out in five selected sites (Fig: 2). A total of 19 populations of fire salamanders were observed. The sites are as follows.

1. Northern region of Prague: It is close to the Vltava River. The observed populations live in small forest fragments surrounded by urban areas with steep valleys and high canyons.
2. Southern region of Prague: This part exhibit similar characteristics as northern Prague with a gradual steep valley. Visible populations exist on both side of the river.
3. Ústí nad Labem (on-going study): Ore mountainous landscape, nearly 31% of forest cover. It falls in the proximity of this sub-urban areas which is divided by the Labe River and surrounded by building areas.
4. Berounsko: Hilly landscape, deep valleys, situated in Křivoklátsko PLA protected landscape area. Populations can be observed in most of the tributaries of the Berounka river on both riverbanks.
5. Posázaví (Chocerady in Fig. 2): Steep fragmented slopes with deep side-valleys in certain areas. Major forests such as oak and beech with Sázava river's tributaries. Chances to observe salamander populations on both sides of the river.

All sites exhibit almost similar level of elevations. The urban populations of fire salamanders were represented by the Northern and Southern region of Prague and Ústí nad Labem as they have high/moderate number of settlement areas, roads, parks, and gardens (n = 11). Whereas populations from Berounsko and Posázaví (n = 8) are considered as a rural site as they have better forest cover with less settlement areas.

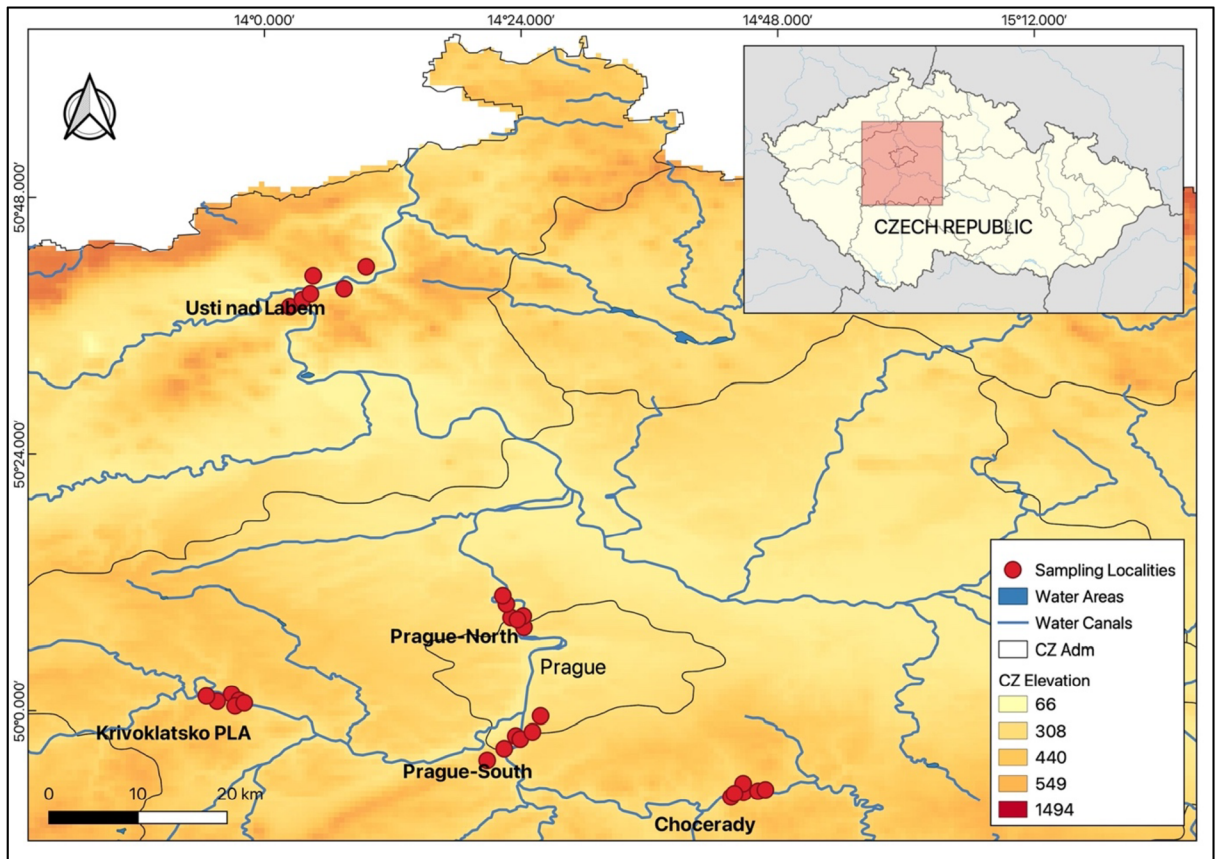


Fig. 2 Elevation map with sampling localities in the vicinity of Prague

5 METHODOLOGY

5.1 DNA EXTRACTION AND MICROSATELLITE ANALYSIS

A random collection of fire salamander (5-30 samples of selected populations) was done in which DNA samples (buccal swabs) were taken by using sterile cotton swabs and kept in a small plastic tube and labelled for every individual.

All samples were kept in a freezer (-18°C) to ensure maximum DNA quality. DNA was extracted from the buccal swabs using the DNeasy Tissue Kit (Qiagen) following the manufacturer's protocol with slight modifications. All samples were genotyped at 14 microsatellite loci named as SST-A6-II, SST-B11, SST-C2, SST-C3, SST-G6, SST-E11, Sal3, Sal 23, Sal E29, Sal E2, Sal E6, Sal E7, SalE8, and SalE12 (Steinfartz et al. 2004, Hendrix et al. 2010). Two tetraplex namely (1. Sal E6, Sal 3, SST-C2, Sal E8; 2. Sal 23, Sal E2, Sal E7, SST-C3) and two triplexes (1. SST-E11, Sal E29, and SST-B11 2. Sal E12, SST-A6-II, and SST G-6) were used for PCR amplification. All multiplex reactions were amplified in a total reaction volume of 10 ul, containing 1ul of DNA with 0.1 to 0.5 ul of each primer (10 ul conc.), 5 ul of PCR buffer, and 1 ul of Q- solution. PCR amplification was conducted using the following conditions: an initial denaturation step of 15 min at 95°C followed by 30 cycles of the 30s at 94°C, 90s at 55°C (at 64°C for triplex), 1 min at 72°C, and a final extension of 30 min at 60°C. PCR products were resolved by capillary electrophoresis on ABI Prism 3130 and analysed using Gene Mapper 3.7 software.

5.2 STATISTICAL DATA ANALYSIS

I genotyped 207 samples from 14 microsatellites and for each microsatellites loci it was ensured for the possible occurrence of null alleles by using CERVUS 3.0.77 (Kalinowski et al. 2007) software in all populations. Further, shift from Hardy-Weinberg equilibrium was tested with Fstat.

I calculated the population genetic parameters such as mean number of allele (A), observed heterozygosity H_0 , expected heterozygosity H_E , and index of inbreeding F_{IS} (Deviations from Hardy-Weinberg expectations) were estimated using the R 3.6.1 software (R core team 2021) with the package *diveRsity* (Keenan et al. 2013). To compare genetic diversity between populations of different sample size allelic richness (Ar) was calculated by rarefaction technique with the *HP-RARE* software (Kalinowski

2005). The difference in genetic diversity (A_r) between urban and rural populations was tested by ANOVA (Girden 1992). ANOVA was also used to test the differences in genetic diversity between the northern and southern populations in order to propose a conservation plan for the Prague population.

Besides, genetic differentiation between populations was determined by pairwise F_{st} and principal coordinates analysis (PCoA) through R software (R core team 2021) and with the help of packages PopGenReport and adegenet (Adamack et al. 2014, Jombart 2008). To determine the proportions of the genetic variance due to differences within and among populations, the genetic variance was hierarchically assigned according to the region using the analysis of molecular variance (AMOVA) using package pegas (Paradis 2010) in R software. This method calculates a standard analysis of variance, in which the explained variance is described in three levels i.e., group-wise, population-wise, and individual-wise.

To test the differences between the populations in urban areas, I used the Bayesian test using STRUCTURE 2.1 (Pritchard et al., 2000) program to find out the number of genetic demes present in the samples. The program STRUCTURE with admixture model was used to find out the genetic clustering within individuals with a burn-in period of 50,000 and MCMC of 250,000 and 5 repetitions of analysis. The selection of optimal K was done based on the Harvest results keeping in mind the Ln probability of data and Delta K plot. The best-fitting model was selected following Evanno, Regnaut & Goudet (2005). Further, program CLUMPP 1.1.2 (Jakobsson and Rosenberg, 2007) was used to ensure that all iterations are combined into one average and the visualization of individuals to clusters was done from the results of DISTRUCT software (Rosenberg 2004).

6 RESULT

6.1 GENETIC DIVERSITY

Overall, 173 individuals with 10 loci's were genotyped (Tab 1). Due to high proportion of null alleles (>10%) 4 loci's (SAL 3, SST-C2, SAL E2, and SST-C3) were deviated from Hardy-Weinberg equilibrium, hence they were excluded from further analysis. The highest allelic richness was observed in the population 16 (Ústecko, $Ar = 4.68$) whereas the lowest allelic richness was represented by population 1 (PRG-NORTH, $Ar = 1.97$) with the mean of 2.97. The expected (H_e) and observed (H_o) heterozygosity's per population ranged from 0.34 to 0.68 and 0.34 to 0.73, respectively. A significant inbreeding coefficient (F_{is}) values were observed in the populations 7 (0.003; PRG-SOUTH), 10 (0.043; PRG-SOUTH) and 11 (0.017; BEROUNSKO) whereas rest of the populations were not significant to the inbreeding coefficient.

Apart from this, the difference in genetic diversity between urban and rural population through ANOVA test (Fig: 6) shows no significant difference ($F = 0.608$, $df = 1$, p -value = 0.446). However, in case of comparison of Prague populations, northern populations showed significantly lower genetic diversity ($F = 24.4$, $df = 1$, p -value = 0.0003).

6.2 POPULATION GENETIC STRUCTURE

The results from the Bayesian clustering method implemented in STRUCTURE has not shown clear number of clusters, (Fig: 4). K ranged from 2 and 3. Whereas the ΔK indicates 2 clusters, $\ln P(K)$ showed 3 clusters. At $K = 2$, the northern population belongs to one cluster and rest of the population to their respective clusters. At $K = 3$, there is a distinct separation of the population 1 (PRG-North) cluster from the northern populations. However, at $K = 4$, population 6 (PRG-North) a noticeable deviation from within this cluster. At $K = 5$, a clear division of ÚSTECKO population from the neighbouring clusters was observed. Interestingly at every level, the population 1 (PRG-NORTH) maintained its distinctness.

Pairwise F_{ST} values ranged from 0 to 0.23 among all 19 populations, with an average $F_{ST} = 0.09$ (Tab 3). These values represent low to moderate levels of population differentiation. The F_{ST} data shows that the urban areas (Mean $F_{ST} = 1.98$) had

moderate genetic differences. Though, the lowest genetic differences were observed in the rural areas (Mean $F_{ST} = 0.0908$). A hierarchical AMOVA (Tab 2) revealed that 74.5% of genetic variation resides within populations, 17.8% are distributed among populations within clusters, and 7.5% of the variance can be explained by the differentiation between the five regional clusters.

The Principal coordinate analysis (PCoA) was calculated in R software to investigate the relative position of populations in multidimensional space. The PCoA individual data results explain 11.05 % (X-axis) of the variation in the data, whereas the Y-axis only explains about 2.95 % (Fig: 3A). In Population, 34.1% (x-axis) of variation was observed and 22.7% (Y-axis) of the genetic variation among populations respectively (Fig: 3B). The PCoA graph for individuals shows that the majority of the individuals were randomly distributed, with only population 1 (PRG-NORTH) displaying a visible cluster. However, it could also be possible due to high number of individuals. The population-wise graph in the PCoA support to the individuals level graph as Prague north populations are visible in one area.

7 DISCUSSION

7.1 GENETIC DIVERSITY

A substantial decrease in genetic diversity may lead to obstruct the evolutionary potential of any species in the changing environment (Hand et al. 2015). Various studies have observed that urban populations of different species such as amphibians and mammals are less genetically diverse than the rural population (Hitchings & Beebee 1997, Noel & Lapointe 2010, Noel et al. 2007). The current study found no significant differences between urban and rural populations of fire salamanders, indicating a low to moderate level of genetic diversity of fire salamander in urban and rural habitats. Though, the comparison between the urban areas (PRG-North, PRG-South) shows a significant difference suggesting a decent genetic diversity. The observable no significant difference between urban and rural areas suggesting remnant semi-natural landscape (vegetation, water channels, suitable management of urban forests) features in the urban areas which might be suitable habitat fire salamanders. However, the significant differences between urban areas imply that the southern populations of fire salamanders are linked to the rural population from the south, while the northern population represents an isolated area. Several studies such as Pisa et al. (Northern Italy, 2015), Fusco et al. (New York city 2020), and Straub et al. (Salzburg, Austria 2015) had also observed nearly similar range of allelic richness, observed and, expected heterozygosity's providing a baseline of genetic diversity in urban areas. Various analysis in the current study shows that urban fire salamander had fair genetic connectivity than the rural areas and it agrees to the studies such as Northern two-lined salamanders (*Eurycea bislineata*) and Fire salamander (*Salamandra salamandra*) in the city areas like New York, and Oviedo (Spain) respectively, which predicted that increase in urban areas will alter gene flow between population, loss of genetic diversity and connectivity; yet they observed a smaller effective population size in the urban area (Lourenco et al. 2017, Fusco et al. 2020). Contrary to the current study predictions that urbanization and loss of connectivity did not affect the overall level of genetic diversity across habitats. It shows that the gene flow was limited in the northern region of Prague which could be due to the presence of settlement areas as it could hinders the connectivity between populations. Apart from this, a significant inbreeding coefficient (Fis) values were observed in the some of the populations and interestingly, the high allelic richness and negative Fis value in population 10 (PRG-SOUTH)

contradict this for the inbreeding coefficient, which could be the result of sensitivity in the sampling size. However, the study also observed a smaller effective populations size in the urban areas which is on the verge of future potential threats to maintaining their genetic diversity.

Within amphibian populations, movement and dispersal are critical for the dynamics of spatially structured populations, affecting demographic inter-dependence and local population growth (Cayuela et al. 2020). The amphibian populations within the metapopulation is considered to occasional long distance movement of >1km (Smith and Green 2005). Some terrestrial salamanders which live in small patches of urban forest (e.g., city parks and gardens), provide the necessary resources (rocks, woody debris, and rotten logs) for species that do not require an aquatic larval stage (Alvarez et al. 2015, Noel & Lapointe 2010). Whereas, terrestrial salamanders with an aquatic larval stage may be able to survive in urbanized catchments if they have enough tree cover, shelter, and foraging microhabitats, as well as waterbodies, to support larval development and metamorphosis (Price et al. 2011, Vanek et al. 2019). However, there could be a significant genetic difference within the fragmented urban populations. Similarly, the observed genetic differences in the current study show that fire salamanders populations can persist in an urban matrix, but dispersers that connect to the other populations were limited by urbanization. Though, the general migration of fire salamander is considered around < 200-400 m (Catenazzi 1998, Shirli et al. 2007; Kiss et al. 2021,) it can be assume that the observed low to moderate level of genetic diversity in the study areas could be due to isolation.

From the past years, the development of urban areas has reached a certain level which turned into a hazard not only for the salamanders but also for different species (Seto et al. 2012, Grimm et al. 2008). Along with this, settlement areas, roads also disturb the surrounding areas via the increase in noise and pollutants leading to a reduction in the densities of the species (Red-backed salamander; Marsh and Beckman 2004. Fahrig et al. 1995). As a result, a possible explanation for the moderate genetic diversity in some parts of the current study; the species could be isolated from the core population (no gene flow), resulting in increased genetic drift and allele loss. However, it has not been tested in this study.

7.2 POPULATION STRUCTURE

Natural habitat fragmentation and its negative effects on species populations do not require extreme urbanization. Moderate suburban development reduces canopy cover and raises stream water temperatures, leaving patches of unsuitable terrestrial habitat interspersed among undeveloped, semi-natural areas (Holgerson et al. 2018). In contrast to this study predictions, the difference in the genetic structure of fire salamander populations in urban and rural areas, show that 19 populations of fire salamanders have moderate genetic differentiation which is also supported and stated low to moderate by studies such as Yannic et al. (2021), Fusco et al. (2020), Munshi et al. (2013). Semlitsch (1998); Porej et al. (2004); Regosin et al. (2005) had mentioned that the quality and quantity of terrestrial habitats around the breeding sites should be important to support amphibian populations. However, several studies have now shown that urbanization increases genetic differentiation between populations in many non-commensal species (Johnson & Munshi-South, 2017). The findings of this study on fire salamander in urban and rural areas support Miles et al. (2019) prediction that urbanization impedes gene flow, resulting in greater genetic differentiation between populations.

Several studies, which shows that roadways also influence genetic structure in Japanese brown frogs (*Rana japonica*) (Kobayashi et al. 2018), and housing, industry, and roadways act as barriers to gene flow in Australia's endangered growling grass frog (*Litoria raniformis*) (Hale et al. 2013). Distance and roads, according to Richardson (2012) and McCartney-Melstad et al. (2018), shape the genetic structure of wood frogs (*Lithobates sylvaticus*) and Eastern tiger salamanders (*Ambystoma tigrinum*), which are often sympatric with *E. bislineata* in north-eastern North America. However, in the current study the sample size was small and urban areas along with barriers such as roads, rivers (Vltava River) could be the possible barriers in the gene flow of fire salamander conversely affecting the connectivity between urban and rural areas. Therefore, it could be possible that the large population of fire salamanders exists at the different/permanent study sites, not because it is close to the human settlement areas. I assume that the population would be insignificant quantity and quality at the study sites if there were no large urban settlements. For example, Sarrikivi et al. (2013) in southern Finland observed the high genetic diversity of common frog (*Rana temporaria*) in 5 golf course areas with very low genetic

differentiation resulting in a better gene flow between sites. Similar results were also observed by Straub et al. (2015) at the pre-alpine urbanized areas of Salzburg (Austria), suggested highways were affecting the gene flow of fire salamanders.

Anthropogenic barriers such as the ocean, rivers, buildings, roads, settlement areas, land use type have an observable effect on many species (Balkenhol et al. 2015, Sunny et al. 2021, Doherty & Driscoll 2018). Voros et al. (2016), Beninde et al. (2016) considered large water systems are not favourable sites for salamanders with difficulties in crossing rivers and affecting their genetic differentiation within sites. Whereas in the current study results demonstrate that some populations are unique (pop 1 (PRG-North) (Fig: 4)) and probably supported by Vltava river to maintain its connectivity and population structure. This result is also supported by the Yannic et al. (2021) landscape hypothesis in which they observed that two large rivers were supporting the overall genetic variation of the fire salamander.

The emergence of the pathogenic infectious fungus (Bsal) has also contributed its part to decrease the populations of fire salamander in countries like Netherland, Germany and other European regions (Slujis et al. 2013). More than 50 Bsal sites have been discovered in the pathogen's invasive European range in the last decade and majority of them are observed in Germany (Lotters et al. 2020). To date the fungus effects has been known to many salamanders and newt (Slujis et al. 2013). As European fire salamanders are vulnerable to the salamander plague and when Bsal infects a population, it usually results in a rapid decline due to mass mortality. However, Bsal was not found in non-systematic field surveys in Australia, Croatia, the Czech Republic, France, Italy, Montenegro, Poland, Slovakia, Slovenia, and Switzerland, implying that it is likely absent in these countries (Parrott et al. 2017, Baláž et al. 2018, EFSA AHAW et al. 2018, Grassel- li et al. 2019, Lastra González et al. 2019, Thomas et al. 2019). Though, recent article had shown that it is not observe in the Czech Republic but it is a serious concern for the populations of fire salamanders in the Czech Republic and the simulation of the Bsal spread should be studied further.

7.3 POSSIBLE FUTURE CONSERVATION STEPS

Overall, most of the studies were carried out in the natural habitat and very few had considered urban areas to explain the genetic structure of fire salamander and related species. The literature review had helped me to find out proposed conservation steps and the appropriate conservation efforts are suggested by Munshi et al. (2014), Saarikivi et al. (2013), Furman et al. (2016), Fidino et al. (2020) and their suggestions in the form of maintaining adequate amount of greenery (>50 ha forest fragment) and water bodies in the urban areas (Maintaining city green space) to balance the genetic connectivity of species. Though, this study had observed a more or less stable populations of fire salamanders along with the areas where gene flow is restricted by settlement areas or roads. In future, this observation can also play a crucial role to at least minimize the level of disturbance resulting in decent gene flow of fire salamanders. Apart from this furthers research in this field is required to make better conservation plans as the urban areas of Prague city still carries a part of existing landscape in which fire salamanders coping to manage its survival.

Still, this study is a step that explored a smaller population size in the urban areas of Prague city which are on the edge of maintaining their survival.

8. CONCLUSION

This small study is a step towards the conservation of fire salamanders in the urban areas and it observe some major points such as:

- No significant genetic diversity had observed between urban and rural population probably due to the semi-natural areas might be suitable habitat for the fire salamanders.
- Certain areas, such as PRG-North, have been observed to have distinct population of fire salamanders, indicating an isolated population.
- The genetic differentiation between the populations was found to be low to moderate. However, when compared to rural areas, urban sites exhibit high genetic differentiation, most likely due to settlement areas and the Vltava River.
- Urban expansion along with different barriers (anthropogenic, Bsal) will alter the evolutionary trajectory and viability of observed populations by reducing gene flow between urban and rural areas. More research and better conservation management are required for the observed viable populations of fire salamander.

RESULTS TABLES:

Tab 1: Genetic diversity within 19 populations of Fire Salamanders in the CR. N, number of genotyped individuals (Sex; F-Female, M- Male, SA- Sub-Adult, U- Unidentified); A, mean number of alleles per locus; Ho observed heterozygosity; He, expected heterozygosity; Fis, index of inbreeding; HWE, Hardy-Weinberg equilibrium; Ar, allelic richness.

S. NO.	Id	N (F/M/SA/U)	Regions	He	Ho	A	Fis	HWE	Ar
1	CR	17 (6/10/1/0)	PRG-NORTH	0.34	0.34	1.86	-0.02	0.946	1.97
2	LH	12 (6/5/1/0)	PRG-NORTH	0.4	0.49	2.17	-0.22	0.549	2.42
3	SA	11 (4/7/0/0)	PRG-NORTH	0.36	0.42	2.07	-0.15	0.439	2.28
4	SB	5 (1/2/2/0)	PRG-NORTH	0.35	0.42	2.01	-0.19	1	2.3
5	SD	6 (1/4/1/0)	PRG-NORTH	0.4	0.43	2.19	-0.1	0.417	2.51
6	DR	9 (5/4/0/0)	PRG-NORTH	0.45	0.51	2.27	-0.13	0.998	2.47
7	KR	16 (8/3/5/0)	PRG-SOUTH	0.65	0.59	3.1	0.081	0.003	3.61
8	VSD	7 (2/2/3/0)	PRG-SOUTH	0.63	0.73	3.05	-0.16	0.118	3.5
9	ZB	6 (0/6/0/0)	PRG-SOUTH	0.44	0.48	2.23	-0.1	0.129	2.53
10	JL	11 (4/6/1/0)	PRG-SOUTH	0.6	0.61	2.78	-0.01	0.043	3.42
11	JV	9 (2/5/1/1)	BEROUNSKO	0.47	0.5	2.45	-0.07	0.017	3.03
12	RA	8 (4/3/1/0)	BEROUNSKO	0.48	0.45	2.39	0.064	0	2.77
13	SY	8 (4/3/1/0)	BEROUNSKO	0.51	0.53	2.6	-0.03	0.976	3.01
14	ZM	10 (3/6/1/0)	BEROUNSKO	0.58	0.57	2.94	0.015	0.549	3.47
15	PS	12 (4/8/0/0)	PŘÍBRAMSKO	0.52	0.48	2.58	0.082	0.996	2.96
16	UNL	7 (4/2/0/1)	ÚSTECKO	0.68	0.7	3.62	-0.03	0	4.68
17	KO	10 (4/6/0/0)	POSÁZAVÍ	0.55	0.57	2.7	-0.04	0.474	3.16
18	KRA	5 (3/1/1/0)	POSÁZAVÍ	0.5	0.54	2.61	-0.08	0.989	3.2
19	KZ	4 (0/4/0/0)	POSÁZAVÍ	0.52	0.65	2.74	-0.25	0	3.3

Tab 2: Analysis of Molecular Variance (AMOVA)

S. no.	Explained Variance	P-value
1	Group	7.566029
2	Population	17.858156
3	Individual	74.575814

Tab 3: Pair-wise Fst between Fire Salamander populations in the study areas.

POP	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
1		0.19	0.24	0.19	0.11	0.09	0.14	0.12	0.2	0.13	0.15	0.12	0.14	0.11	0.15	0.15	0.12	0.15	0.15
2			0.09	0.08	0.12	0.09	0.1	0.1	0.18	0.08	0.11	0.06	0.07	0.07	0.17	0.09	0.07	0.07	0.07
3				0.03	0.12	0.12	0.1	0.12	0.13	0.08	0.16	0.12	0.11	0.12	0.19	0.1	0.1	0.08	0.12
4					0.15	0.13	0.07	0.13	0.13	0.07	0.14	0.12	0.08	0.09	0.15	0.1	0.1	0.11	0.16
5						0.11	0.07	0.1	0.18	0.08	0.14	0.09	0.13	0.09	0.12	0.1	0.08	0.1	0.13
6							0.06	0.07	0.14	0.06	0.08	0.07	0.08	0.07	0.11	0.09	0.07	0.11	0.11
7								0.03	0.06	0.05	0.07	0.06	0.04	0.05	0.08	0.04	0.05	0.05	0.05
8									0.1	0.03	0.07	0.07	0.07	0.05	0.06	0.06	0.04	0.06	0.05
9										0.07	0.13	0.16	0.08	0.12	0.12	0.11	0.1	0.14	0.13
10											0.06	0.06	0.05	0.05	0.06	0.04	0.04	0.05	0.04
11												0.05	0.06	0.04	0.07	0.08	0.07	0.09	0.07
12													0.06	0.03	0.09	0.08	0.05	0.06	0.06
13														0.03	0.09	0.08	0.05	0.07	0.07
14															0.07	0.06	0.04	0.05	0.06
15																0.09	0.09	0.09	0.08
16																	0.06	0.08	0.07
17																		0.03	0.04
18																			0.05
19																			

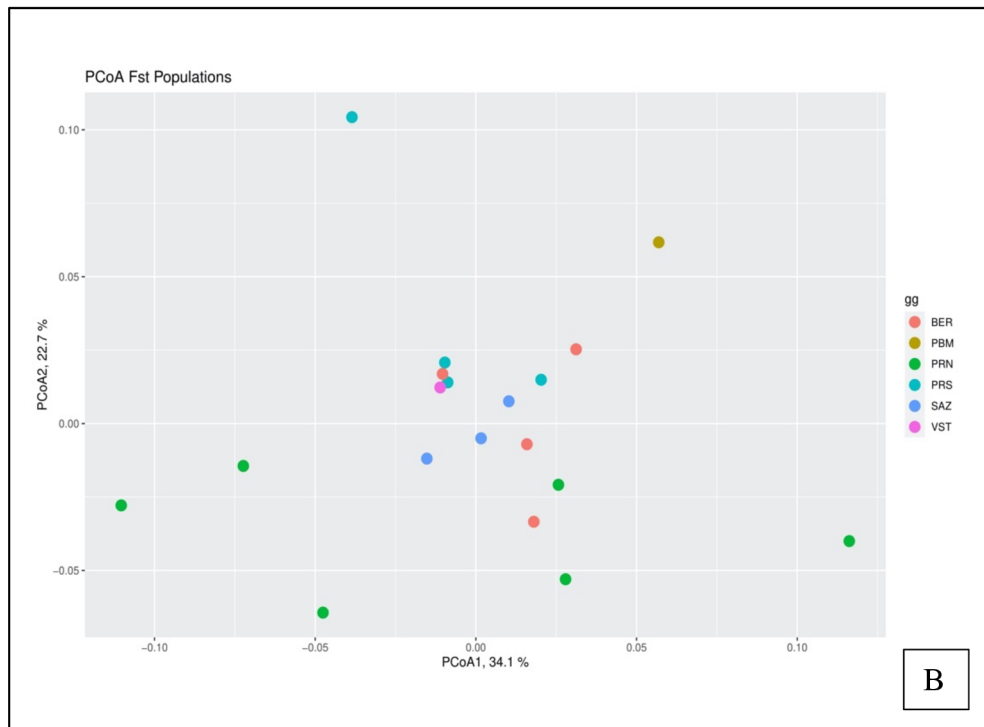
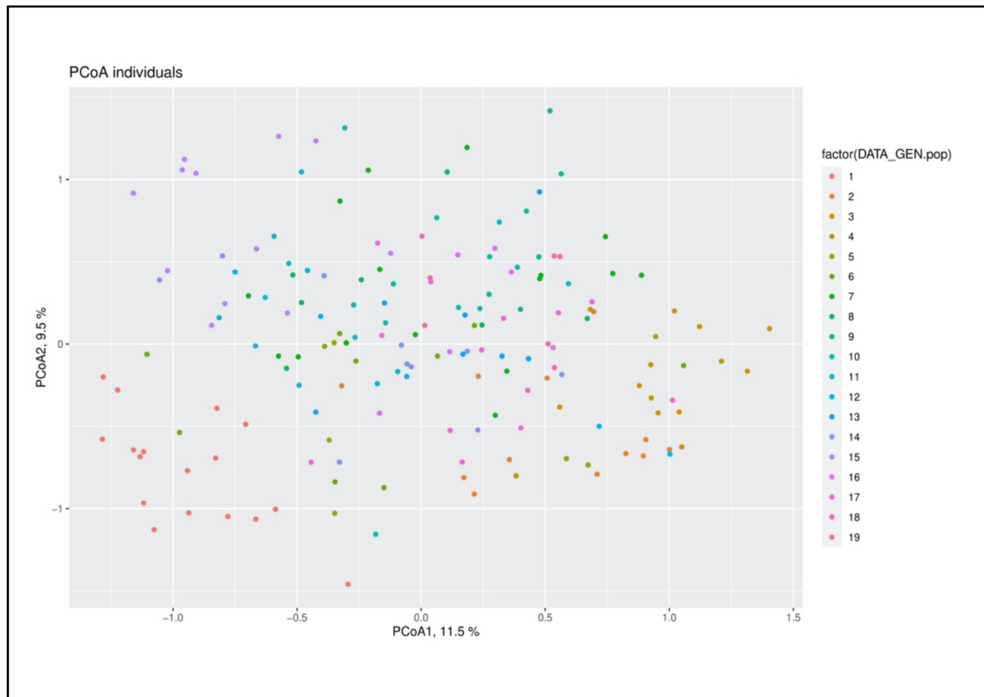


Fig: 3 Principal Coordinate Analysis; A- Represents the PCoA analysis for 19 individuals (Individual-wise); B- Represents the PCoA for 19 Population. PRGN = Prague North, PRGS = Prague South, BER = Berounsko, PBM = Příbramsko, VST (Correct UST*) = Ústecko, SAZ = Posázaví

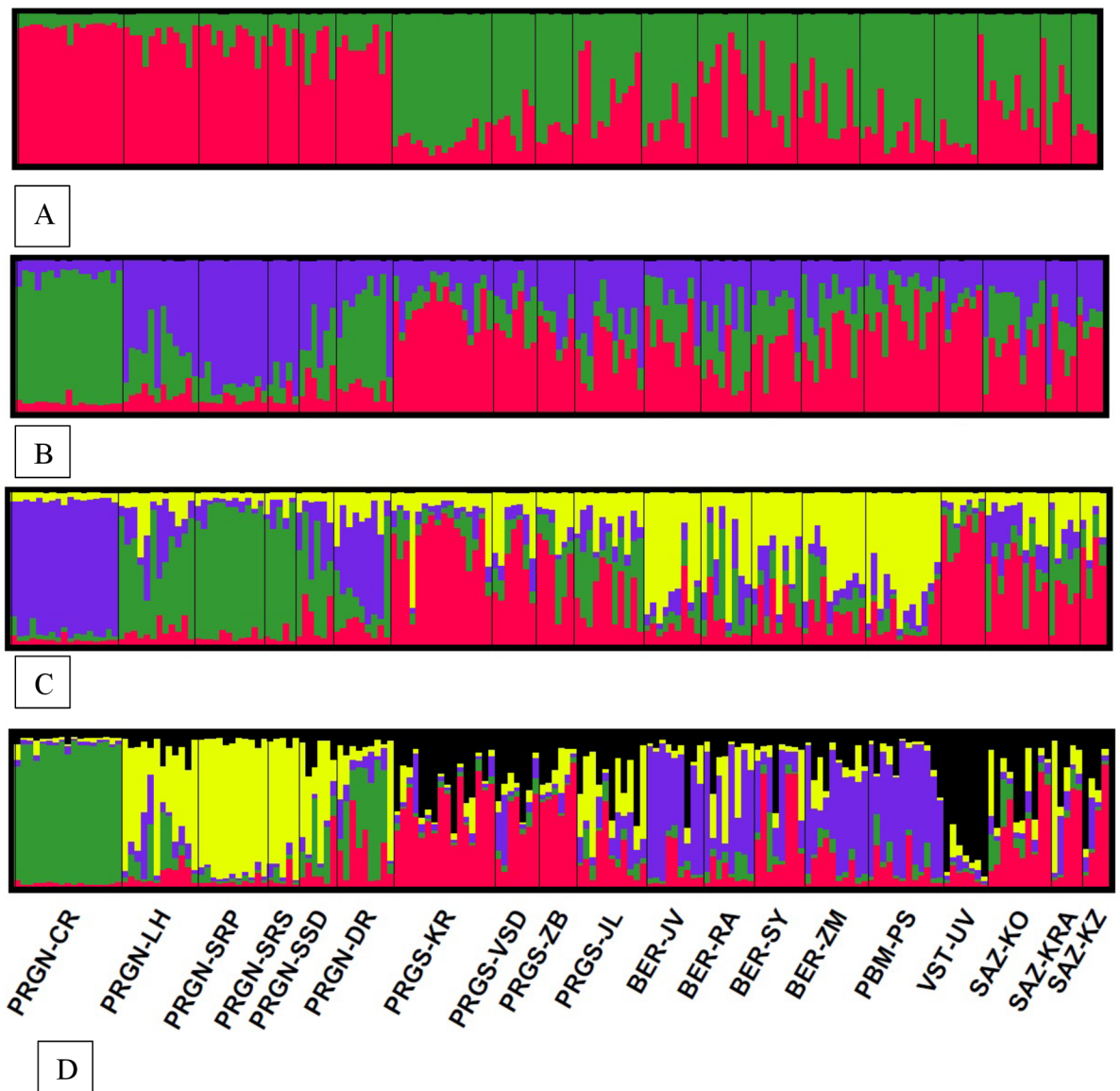
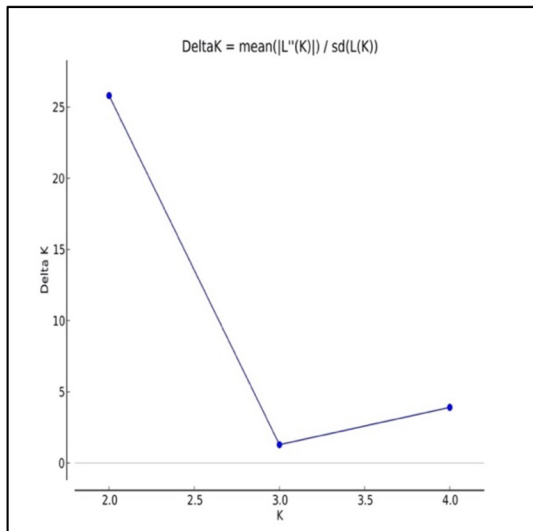
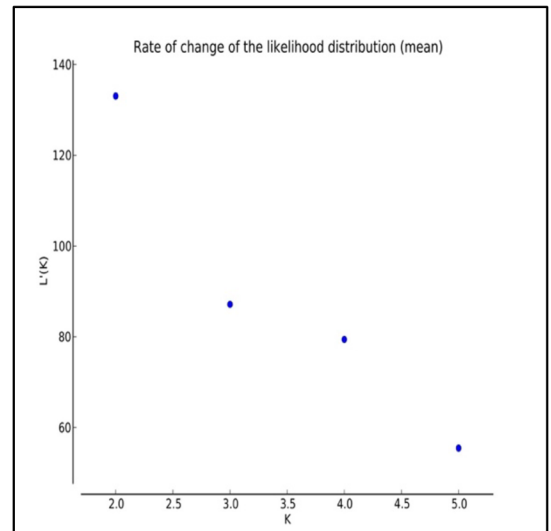


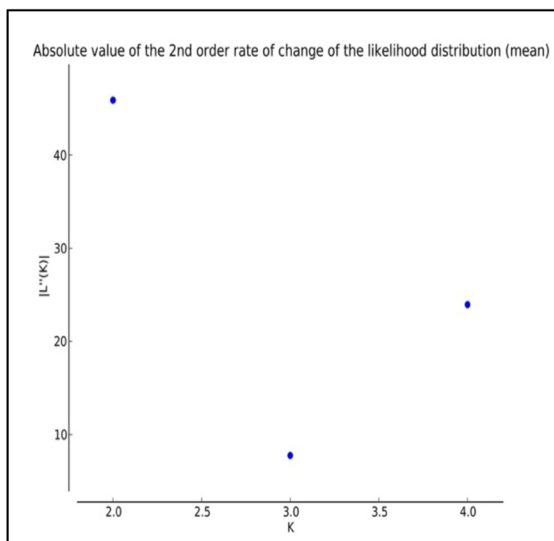
Fig: 4 Bayesian assignment Probabilities for A: K=2 and B: K=3, C: K=4, D: K=5. Figure was generated in STRUCTURE software.
 19 populations: PRGN = Prague North, PRGS = Prague South, BER = Berounsko, PBM = Příbramsko, VST (Correct UST*) = Ústecko, SAZ = Posázaví



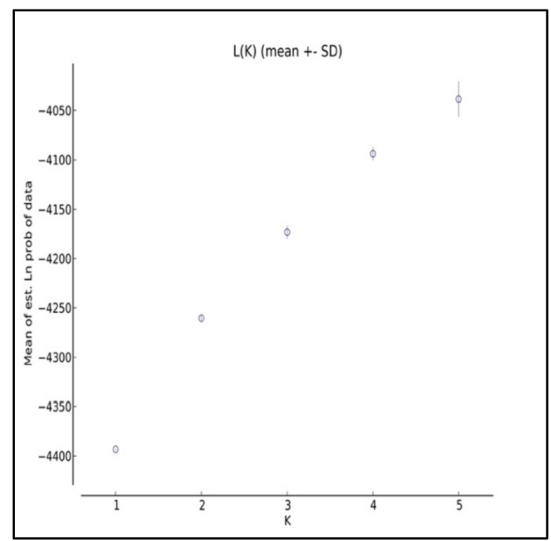
A



B



C



D

Fig. 5 Graphs represents the outcomes from the harvest software.
 A- represents the Delta k value= $\text{mean} [|L''(K)|] / \text{sd}(L(K))$
 B- represents the rate of change of the likelihood distribution (mean).
 C- Represents the absolute value of the 2nd order rate of change of the likelihood distribution.
 D- Represents the L(K) (mean \pm SD).

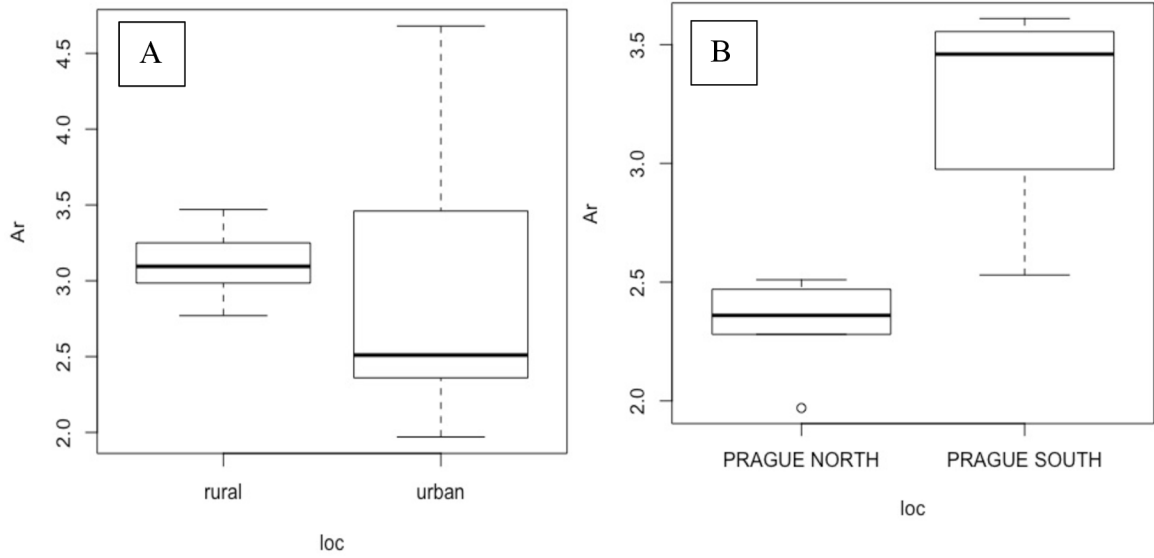


Fig 6. A, represents the difference in genetic diversity between urban and rural populations. B, represents the difference in genetic diversity between northern and southern populations in Prague

Field Photos of Fire Salamanders:



Fig 7. Fire Salamanders (*S. salamandra*) in the city of Prague.

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10. APPENDICES

Tab: 4 Table of Empirical studies.

S. No.	year	Species	Authors	Place	Sample type	Sample no.	Marker	Method	Software	Amplification
1	2007	Eastern red-backed salamander	Sarah Noe ¹ Æ Martin Ouellet Æ Patrick Galois Æ François-Joseph Lapointe	Montreal (Quebec, Canada),	Tissue Sample (Tail tip)	221 indi	7 Microsatellites	Standard Phenol-chloroform protocol	FSTAT 2.9.3, Genepop v 3.2, Fisher exact test	
2	2009	Italian salamanders <i>Salamandrina perspicillata</i> (Savi, 1821) and <i>S. terdigitata</i>	A. ROMANO, M. MATTOCCIA, S. MARTA, S. BOGAERTS, F. PASMANS, & V. SBORDONII	Central Italy	Tissue sample (Tail tip)	56 sampling sites	3 Mitochondrial DNA genes		CART (Classification and Regression Tree Analysis)	12S and 16S ribosomal RNAs and cytochrome <i>b</i>
3	2010	Red-backed Salamander (<i>P. cinereous</i>)	Sarah Noe & François-Joseph Lapointe	Montreal Island, Eastern North America	Tissue Sample (Tail tip)	10 populations (28-48 indi/pop)	6 dinucleotide Microsatellites	Phenol-chloroform protocol	Genepop 3.2, Bottleneck 1.2, structure 2.2	
4	2010	white-footed mouse (<i>Peromyscus leucopus</i>)	JASON MUNSHI-SOUTH and KATERINA KHARCHENKO	New York City	tail snips	312 indi	18 Microsatellites	Qiagen DNeasy kits	FSTAT 2.9.3, BAPS and STRUCTURE 2.3	
5	2011	northern dusky salamander (<i>Desmo-gnathus fuscus</i>) and southern two-lined salamander (<i>Eurycea cirrigera</i>)	STEVEN J. PRICE*, †, ROBERT A. BROWNE† AND MICHAEL E. DORCAS*	Charlotte-Metropolitan area of North Carolina, U.S.A	Annula occupancy check	6558 dusky and two-lined salamanders between 2005 and 2009		linear transects (occupancy modelling)	GIS, Arc GIS	
6	2012	<i>Salamandra infraimmaculata</i>	L. Blank ¹ , I. Sinai ^{1,2} , S. Bar-David ^{1,3} , N. Peleg ¹ , O. Segev ¹ , A. Sadeh ¹ , N. M. Kopelman ⁴ , A. R. Templeton ^{1,5} , J. Merilä ⁶ & L. Blaustein ¹	Northern Israel (Galilee and Mt. Carmel region)	Tissue Sample (Tail tip)	475 Adults	10 Microsatellites	QIAamp DNA Mini kit	FSTAT 2.9.3, Microchecker 2.2.3 and STRUCTURE 2.3	
7	2012	marsh frog (<i>Pelophylax ridibundus</i>)	Peter Mikulíček & Peter Pišút	Bratislava city, Slovakia	Tissue Sample (Toe clipping)	494 indi	11 Microsatellite	Qiagen Multiplex Kit, Bayesian	GenAIEx 6.1, FSTAT 2.9.3, Microchecker 2.2.3	
8	2012	white-footed mouse (<i>Peromyscus leucopus</i>)	JASON MUNSHI-SOUTH*†	New York City	tail snips	294 indi	18 Microsatellites	Qiagen DNeasy blood and tissue kit	FSTAT 2.9.3, BayesAss+ 1.3 and STRUCTURE 2.3, ArcGis 9.3	
9	2012	Fire Salamander (<i>S. salamandra</i>)	G Velo-Anto ⁿ 1,2,3, KR Zamudio ¹ and A Cordero-Rivera ²	San Martín o and Ons islands are part of the Galician Atlantic	Tissue Sample (Tail tip/Toe clipping)	32 tissues/locality	8 Microsatellites	Lithium chloride protocol	Genemapper 3.7, Microchecker 2.2.3, Genalex v.6, Structure 2.3.1	

			Islands		National Park							
10	2013	Nothern Dusky Salamander (<i>Desmognahus fuscus</i>)	Jason Munshi-South, Yana Zak and Ellen Pehek	New York City	Tissue Sample (Tail tip)	5 sites (app. 48km)	5 Tetranucleotide Microsatellites	QiagenDNeasy kits	Genepop 4.0, Microcheker, Genalex 6.4, Structure 2.3			
11	2013	Common Frog (<i>Rana temporaria</i>)	J. Saarikivi • T. Knopp • A. Granroth • J. Merila	Southern Finland	Nose Tissue	5 Golf courses	48 Microsatellites	Chelex-100 protocol	2.2.3, FSTAT2.9.3.2, Genepop 4.0			
12	2014	Terrestrial woodland salamander (<i>Plethodon albagula</i>)	WILLIAM E. PETERMAN, GRANT M. CONNETTE, RAYMOND D. SEMLITSCH and LORI S. EGGERT	East-Central Missouri	Tissue Sample (Tail tip)	10 to 25 samples from 22 sites	24 tetra and penta-nucleotide microsatellites	Wizard SV 96 Genomic DNA Purification System	GeneMarker, Genepop			
13	2014	white-footed mouse (<i>Peromyscus leucopus</i>)	Jason Munshi-South and Christopher Nagy2	New York City	tail snips	294 indi	18 Microsatellites	Qiagen DNeasy blood and tissue kit	Bottleneck 1.2, FSTAT 2.9.3, GenAlex 6.2, BOTTLENECK 1.2	324 bp segment of the mitochondrial D-loop		
14	2015	<i>Salamandra s.</i>	Christina Straub, Florian Pichlmüller & Véronique Helfer	Salzburg, Austria	Buccal Swabbing	70 indi	7 Microsatellites	Qiagen DNeasy blood and tissue kit	FSTAT 2.9.3, and STRUCTURE 2.3	<50% success		
15	2015	Fire Salamander (<i>S. salamandra</i>)	David A´ lvarez, Andre´ Lourenc, Daniel Oro Guillermo Velo-Anto	Oviedo	Tissue Sample (Tail tip/Toe clipping)	216 indi	5 microsatellite	Genomic DNA Tissue kit	GeneMapper v4.1, Genepop			
16	2015	Fire Salamander (<i>S. salamandra</i>)	Monika Balogová, Mária Apfelová, Tomáš Flaajs, Daniel Jablonski, Ján Kautman, Peter Krišovský, Anton Krištín	Slovakia		online published data						
17	2016	common wall lizard	JOSCHA BENINDE,* STEPHAN FELDMEIER,* MAIKE WERNER,† DANIEL PEROVERDE,* ULRICH SCHULTE,‡ AXEL HOCHKIRCH* and MICHAEL VEITH	Trier city, Germany	Buccal Swabbing	223 indi	17 Microsatellites	Qiagen DNeasy blood and tissue kit	GenAIEx 6.1, Microcheker 2.2.3, Geneland	cytochrome b		
18	2016	Wood frog (<i>Lithobates sylvaticus</i>)	Benjamin L. S. Furman • Brett R. Scheffers • Murdoch Taylor • Corey Davis • Cynthia A. Paszkowski	Edmonton, Alberta, Canada	Tissue Sample (Toe clipping)	182 frogs	10 Microsatellites	Qiagen DNeasy blood and tissue kit standard	GeneMapper v4.1, Genepop, Bottleneck			
19	2016	Fire Salamander (<i>S. salamandra</i>)	Agnieszka Konowalik, Anna Najbar, Wieslaw Babik, Sebastian Steinfartz, Maria Ogielska	Polish part of the Sudetes	caudal folds-larvae, phalanx of the right hind limbs	1100 indi	11 Microsatellite	standard proteinase K-phenol-chloroform method	GeneMapper v4.1, Microcheker v 2.2.3			
20	2016	<i>Salamandra s.</i>	JUDIT VOEROES1,2, SYLVAIN URSENBACHER3, ISTVAN KISS4, DUSAN JELIC5, SILKE SCHWEIGER6 and KRISZTIAN SZABO7	Carpathian Basin	Tissue Sample (Tail tip)	349 indi	10 Microsatellites	Qiagen Dneasy Blood and Tissue kit	FSTAT 2.9.3, and GENALEX 6.501, Structure2.3.1	725-bp fragment		

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