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RESEARCH PAPER

Variability of colour pattern and genetic diversity of *Salamandra salamandra* (Caudata: Salamandridae) in the Czech Republic

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Abstract. Two evolutionary lineages of the fire salamander occur in central Europe: the typically striped subspecies *Salamandra salamandra terrestris* (Bonnaterre, 1789) and the typically spotted *Salamandra salamandra salamandra* (Linnaeus, 1758). In the Czech Republic, fire salamanders have traditionally been viewed as belonging to the *S. s. salamandra* evolutionary lineage. Nevertheless, the colour pattern of some individuals in the westernmost part of the Czech Republic resembles that of *S. s. terrestris* in having parallel continuous bands along the back. In this study, we investigated whether in the Czech Republic the presence of striped fire salamander phenotype could be associated with the genotype of *S. s. terrestris*. We sequenced the mitochondrial D-loop and two nuclear markers, Rag2 and PDGFRα, of 61 fire salamander individuals from the Czech Republic. To describe the geographical distribution pattern of the striped and spotted fire salamander phenotype in the Czech Republic, we evaluated colour phenotypes of 398 individuals from ten localities distributed so as to cover the whole country. We found no evidence of presence of genotypes corresponding to the *S. s. terrestris* lineage. We did, however, find that the striped phenotype is found mostly in the northwest of the Czech Republic, where both the striped and the intermediate phenotype occur significantly more frequently than in the rest of the country, where the spotted phenotype seems dominant. This finding indicates that Czech and Polish populations of *S. salamandra* show a degree of phenotypic pattern variation comparable to that observed in German populations, although at a local level the frequencies of the striped and spotted phenotype vary. It would be interesting to test whether a genetic toolkit responsible for the colour pattern is shared via genetic introgression between populations, or whether the striped phenotype of Czech fire salamanders evolved independently.

Key words: fire salamander, *S. s. terrestris*, coloration, pigment, evolution, biogeography, Bohemian Massif, Central Europe

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Introduction

Coloration can aid animal survival and reproduction in various ways, including protection against solar radiation, crypsis, communication, and recognition of conspecifics (Cuthill et al. 2017). One evolutionarily important function of body coloration is its role in speciation (Andersson & Simmons 2006). For instance, once females develop preference for a particular colour patterns in males, coloration can – much like geographical isolation – function as a prezygotic reproductive barrier (Kirkpatrick & Ravigné 2002). On the other hand, the genetic toolkits responsible for colour and colour patterns can be shared via hybridisation bewtween distinct evolutionary lineages (Dasmahapatra et al. 2012, Taylor & Larson 2019) and can also lead to speciation (Mallet 2007). Identification of colour phenotypes in closely related evolutionary lineages is therefore important in understanding the functional role of coloration in a species.

Although fire salamander (*Salamandra salamandra* Linnaeus, 1758) coloration is limited to brown, black, red, and yellow, different lineages of fire salamanders display a range of distinct colour patterns (Seidel & Gerhardt 2016). Fire salamanders develop their colour pattern, i.e. the form and arrangement of spots and stripes, after metamorphosis (Pederzoli et al. 2003). During the course of their life the colour pattern of fire salamanders from the Balkan peninsula undergoes only subtle changes (Wisniewski & Wisniewski 1998), but some pattern changes have been reported in Slovak populations (Balogová et al. 2016) and the Corsican fire salamander (*Salamandra corsica* Savi, 1838) exhibits pronounced colour pattern changes (Beukema 2011). The colour pattern of fire salamanders in the Czech Republic has been used by researchers to distinguish individuals (Opatrný 1983, Peprný 2000).

In central Europe, we find two evolutionary lineages of *S. salamandra*, which have traditionally been considered distinct subspecies (Veith 1992, Steinfartz et al. 2000, Dufresnes 2019). The banded fire salamander (*Salamandra salamandra terrestris* Bonnaterre, 1789), type locality Normandy (Eiselt 1958), is found from the Pyrenees to Germany. The spotted fire salamander (*S. s. salamandra* Linnaeus, 1758), type locality Nürnberg (Mertens & Müller 1928), is distributed throughout eastern Germany, central and eastern Europe, and the Balkans (Thiesmeier & Grossenbacher 2004, Seidel & Gerhardt 2016). As the vernacular names suggest, representatives of the two lineages differ in their dorsal colour pattern. *Salamandra s. terrestris* have two parallel stripes along their back, while spotted fire salamanders are characterised by an irregular

Fig. 1. Colour pattern phenotypes of fire salamanders (*Salamandra salamandra*) from the studied area. A) the striped phenotype from north-western Bohemia, Czech Republic. B) the spotted phenotype from central Bohemia, Czech Republic (photos Jiří Moravec).

distribution of isolated spots on their back (Boulenger 1911). Boulenger (1911) claimed that there are no transitions or intermediate phenotypes in-between the striped *S. s. terrestris* of western Europe and the spotted *S. s. salamandra* of eastern Europe. Later authors, however, demonstrated that intermediate phenotypes do exist and in fact occur widely in central Europe (Eiselt 1958, Steinfartz et al. 2000, Weitere et al. 2004). According to Arnold (2002), the two lineages share a contact zone, which – based on colour pattern phenotypes – appears to stretch from eastern Germany to the Czech Republic and Poland. To date, only a few studies have attempted to quantify the distribution of fire salamanders' colour phenotypes (Klewen 1985, Beukema et al. 2016, Najbar et al. 2018, Burgon et al. 2020) and even fewer have related it to the distribution of genetic lineages (Veith 1992, Beukema et al. 2016, Najbar et al. 2018, Burgon et al. 2020).

The localisation of a contact zone between the western and eastern evolutionary lineages of fire salamanders, traditionally referred to as *S. s. terrestris* and *S. s. salamandra*, in central Europe remains an open question (Veith 1992, Arnold 2002, Najbar et al. 2018). This is despite the fact Weitere et al. (2004) described two contact zones based on mitochondrial D-loop sequences: one in south-western Germany, the other in north-western Germany. The most recent genome-wide study confirmed the presence of two contact zones in Germany, one in the extreme south, the other in the north of the country, including the Elbe Valley (Burgon 2018, Supplementary Figure A4.6). The northern contact zone is not limited to north-western Germany, it extends across the north towards the northeast but the study did not include any sampling points further to the east so the full extent of the distribution remains unclear (Burgon 2018).

In the Czech Republic, fire salamanders have historically been believed to belong to the evolutionary lineage *S. s. salamandra*. On the other hand, the presence of *S. s. terrestris* has been reported in Germany, near the north-western border of Bohemia, especially in the vicinity of Meißen, Dresden, and Zittau (Baruš & Oliva 1992, Zöphel & Steffens 2002), and striped phenotypes were found over a hundred years ago around Liberec, which is again in the north-western part of Bohemia (Pražák 1898). Phenotypical characteristics of some individuals in the westernmost part of the Czech Republic resemble those of *S. s. terrestris* in that they have parallel, more or less continuous stripes along the back (Baruš & Oliva 1992, Benda 2015, Moravec 2019). In the rest of the country, the spotted phenotype seems dominant (Fig. 1).

Hitherto, we do not have the data on either the exact distribution of colour phenotypes or the genetic variation of *S. salamandra* in the Czech Republic. In this study, we report our findings on the distribution of dorsal colour pattern phenotypes and genetic diversity of fire salamanders in the Czech Republic. Specifically, we ask whether the presence of the striped phenotype could be associated with the genotype of *S. s. terrestris* in the Czech Republic.

Material and Methods

Analysis of distribution of colour pattern phenotypes

To describe the geographical distribution of the striped and spotted phenotypes of fire salamanders in the Czech Republic (current distribution of *S. salamandra* in the Czech Republic, Jeřábková & Zavadil 2020), we collected photographs of 398 individuals (National Museum Praha (NMP-P6V), voucher specimens and living individuals recorded in the field) from ten localities which cover the whole range of the country (Jablečno, Vaňov-Ústí nad Labem, Děčín, Roztoky, Praha-Troja, Kuroslepy, Velká nad Veličkou, Libavá; coordinates in Table 1, Fig. 2). Previous studies on fires salamanders in the Czech Republic showed that colour pattern can be used to discriminate individuals (Opatrný 1983, Peprný 2000), hence no individual marking was needed to avoid re-captures.

Eiselt (1958) divided the colour pattern phenotypes of *S. salamandra* into four categories: striped, striped-spotted, spotted-striped, and spotted. Klewen (1985) and Najbar et al. (2018) expanded this division by adding further categories. Because we were mostly interested in differences between the striped and the spotted phenotypes, we simplified the division and, based on photographs, sorted individual phenotypes into three colour pattern categories following Benda (2015) or Veith (1992). The categories were defined as follows: a) striped: a yellow dorsal pattern consisting of two continuous or mostly continuous dorsolateral bands (Fig. 1A), b) intermediate: a symmetric dorsal pattern consisting mostly of separate spots, some of which may be asymmetric, c) spotted: no obvious continuous symmetric bands can be discerned, the yellow dorsal pattern consists of irregular, randomly distributed spots (Fig. 1B), which can be accompanied by a single continuous asymmetric stripe along the body.

We calculated the ratio of each colour pattern category for each population and interpolated the values for the geographical space of the Czech Republic using the RCzechia package (Lacko 2020) and interpolation function *idw* in the *gstat* package (Pebesma 2004) in R software (R Core Team 2017). Then we compared the counts of colour pattern categories between all populations with Pearson's chi-squared test using chisq.test followed by a post hoc analysis based on the residuals of Pearson's chi-squared test for count data in the chisq.posthoc.test package (Ebbert 2019) in R.

Genetic analyses

Sampling (Fig. 3A, Table S1) was designed to cover the west-east latitudinal gradient of the fire salamander range in the Czech Republic, with focus on the north-western part of the country where individuals of the striped phenotype were previously reported. We collected 77 individuals of *S. salamandra*, 61 of whom were collected specifically for this study in the Czech Republic and four in Slovakia. Collection took place in 2017-2018. Samples were obtained from roadkill. Samples from the core area of distribution of *S. s. terrestris* (two individuals from Germany) were kindly provided by Professor Wolfgang Böhme from collections of the Zoologische Forschungsmuseum Alexander Koenig in Bonn (ZFMK). Samples from the core areas of distribution of *S. s. salamandra* (five samples from Greece-NHMC, three from Albania, one from Ukraine, and one from Slovenia) were obtained from roadkill collected during another study.

Small tissue samples were preserved in 96% molecular grade ethanol. DNA was extracted using the Tissue Genomic DNA Mini Kit (GT300, Geneaid) according to the manufacturer's instructions. To barcode the salamanders, we used three DNA markers: the mitochondrial D-loop and two nuclear markers, Rag2 and PDGFRα, because it has been reported that these are sufficiently variable to enable a comparison between closely related evolutionary lineages (Vences et al. 2014). We used L-PRO-ML and H-12S1-ML to amplify the D-loop, PDGFRa2F and PDGFRa2R-F primers to amplify the PDGFRα, and RAG2-SAL-F2 and RAG2-SAL-R1 to amplify Rag 2 (Vences et al. 2014). PCR was performed in a final volume of 25 µl. PCR conditions were as follows: 120 s at 94 °C for initial incubation; 39 cycles of 20 s at 94 °C, 50 s at 60 °C, 180 s at 72 °C, and a final extension for 10 min at 72 °C. This was followed by PCR product purification (Šanda et al. 2008). Sequencing was carried out by Macrogen Service Centre Europe (Amsterdam, Netherlands) using amplification primers.

From the GenBank database (Sayers et al. 2019), we downloaded the sequences of *Salamandra salamandra gallaica* (D-loop – KX094979.1, PDGFRα – KF645649, Rag2 – KF645724.1) and *Salamandra salamandra longirostris* (D-loop – KF645599.1,

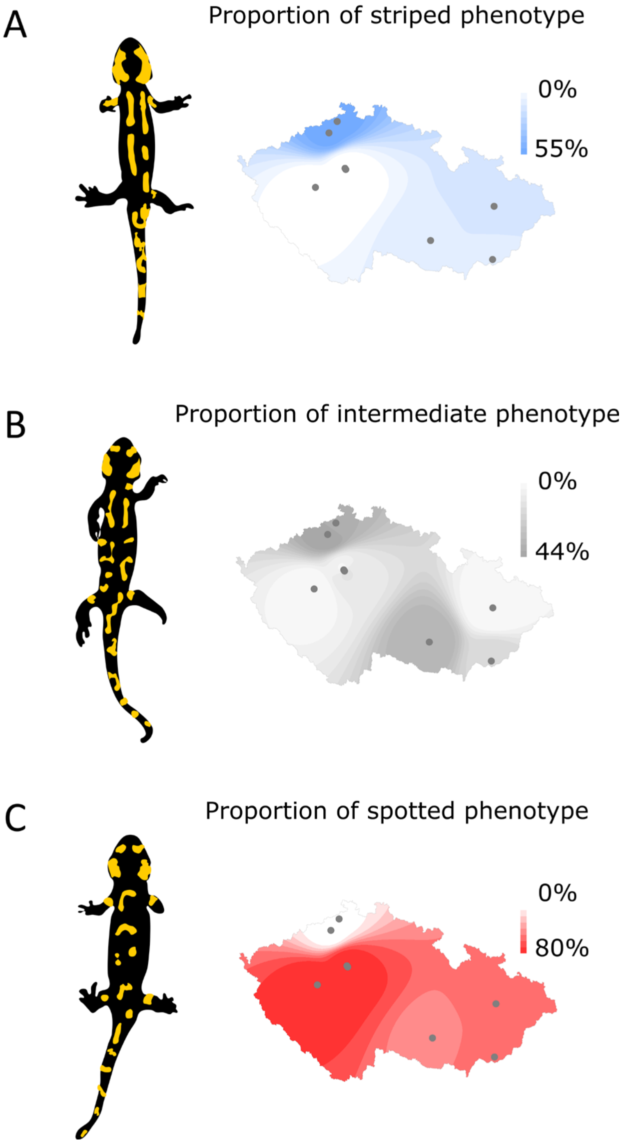

Fig. 2. The distribution of proportions of colour pattern phenotypes of fire salamanders in the Czech Republic as extrapolated in geographical space. A) striped phenotype, B) intermediate phenotype, C) spotted phenotype. Note that the scale for different phenotypes differs.

Table 1. Differences between populations and the rest of the Czech Republic after post hoc analysis based on residuals of Pearson's chi-squared test for counts of individuals belonging to one of the three colour pattern phenotypes.

Locality	Coordinates (WGS84)			Colour pattern		
	N	E	Value	Striped	Intermediate	Spotted
Jablečno	49.88	13.75	proportion of phenotype	0%	20%	80%
n = 10			*P* values	1	1	1
Ústí nad Labem-Vaňov	50.63	14.05	proportion of phenotype	52%	44%	4%
n = 25			*P* values	0	1	0
Děčín	50.79	14.22	proportion of phenotype	53%	40%	7%
n = 15			*P* values	0	1	< 0.001
Roztoky	50.15	14.39	proportion of phenotype	3%	19%	78%
n = 119			*P* values	0.080	0.060	< 0.001
Praha-Troja	50.13	14.4	proportion of phenotype	0%	30%	70%
n = 120			*P* values	< 0.001	1	0.346
Kuroslepy	49.15	16.21	proportion of phenotype	13%	40%	47%
n = 76			*P* values	1	0.720	0.233
Libavá	49.62	17.56	proportion of phenotype	20%	27%	53%
n = 15			*P* values	1	1	1
Velká nad Veličkou	48.89	17.53	proportion of phenotype	12%	29%	59%
n = 17			*P* values	1	1	1

PDGFRα – KF645651.1, Rag2 – KF645726.1) to use as outgroups when assessing the differentiation between *S. s. terrestris* and *S. s. salamandra*. All sequences were checked for quality manually and homologous regions were aligned using the ClustalW algorithm implemented in Geneious 9.0.5. (Kearse et al. 2012). Haplotypes of the mitochondrial D-loop were determined using DNAsp 5.10 (Librado & Rozas 2009) and the haplotype network was calculated in TCS 1.21 (Clement et al. 2002) using 95% connection limit and gap as the fifth character. The network was visualised using tcsBU (Múrias dos Santos et al. 2016). Sequences were concatenated and the neighbour joining tree calculated in Geneious 9.0.5 using the Juke-Cantor genetic distance model and bootstrap resampling (random seed = 467,663, with 10,000 replicates). The partition scheme was determined by Partitionfinder 2.1.1 (Lanfear et al. 2017). A Bayesian inference tree was calculated using MrBayes 3.2.6 (Ronquist et al. 2011). The analysis consisted of two simultaneous runs, four MCMC chains, and ten million generations. The trees were sampled every 100 generations, whereby the first 25% of trees were discarded. Bayesian posterior probabilities (BPP) were estimated from the post burn-in samples, and the 50% majority-rule consensus tree was generated from the retained posterior distribution trees.

Results

Analysis of distribution of colour pattern phenotypes

Table S2 presents a categorisation of individuals based on photographs. A distribution of ratios of colour pattern as well as interpolation of values onto the geographical space of the Czech Republic are shown in Fig. 2. Localities in the north-western part of the country (Děčín and Ústí nad Labem-Vaňov) clearly show an increase in the presence of striped and intermediate individuals and a

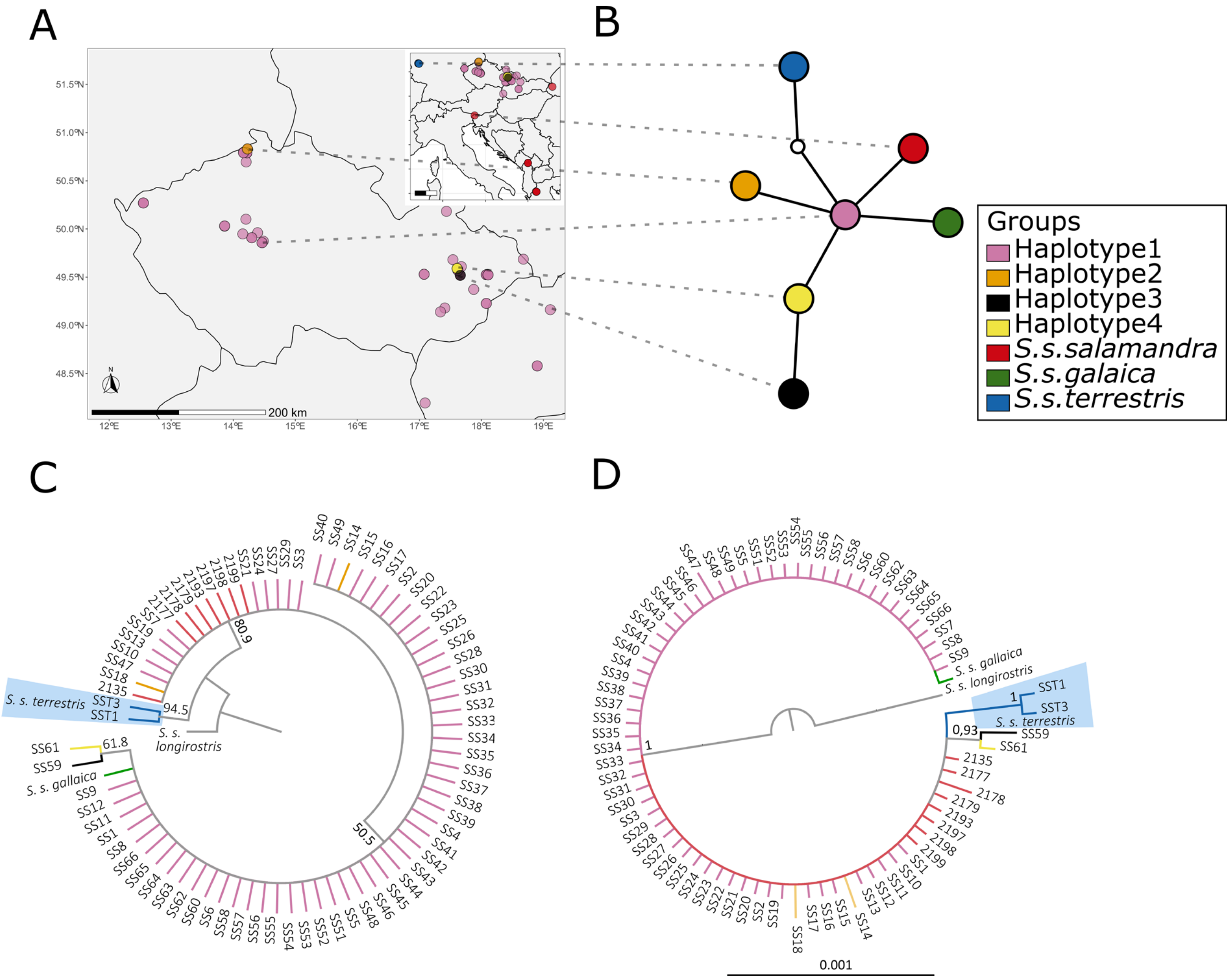

Fig. 3. Sampling design and the results of genetic analyses. A) Sampling design, where circles represent individuals and colour coding represents haplotype membership: blue – *Salamandra salamandra terrestris* from Germany, red – *Salamandra salamandra salamandra* from the Balkan peninsula and Ukraine, reddish purple – fire salamanders from the Czech Republic and Slovakia that bear “haplotype 1”, the most common found in this study, orange – two individuals from Děčín (SS14, SS18) that bear “haplotype 2”, black – individual SS59 from Oderské vrchy that bear “haplotype 3”, yellow – individual SS61 that bears “haplotype 4”. B) A statistical parsimony haplotype network (connection limit 95%) for 699 bp partial D-loop sequence of studied salamanders calculated using the TCS algorithm. In this network, branches represent mutations, small blank circles represent missing hypothetical haplotypes. C) Neighbour joining tree based on a concatenated sequence of mitochondrial D-loop and nuclear Rag2 and PDGFR*α* sequences (1,335 bp), where node numbers show consensus percentage support, while branches are of equal length. Individual samples are colour coded according to haplotype membership. D) Bayesian inference tree based on a concatenated sequence of mitochondrial D-loop and nuclear Rag2 and PDGFR*α* sequences (1,335 bp). Node numbers show probability, the scale bar represents the number of nucleotide substitutions per site, individual samples are colour coded by haplotype membership.

decrease in the occurrence of spotted individuals compared to the rest of the country. The presence of the intermediate phenotype was also increased at locality Kuroslepy and slightly elevated in Velká nad Veličkou and Praha-Troja. The highest occurrence of the spotted phenotype was observed in Jablečno, Praha-Troja, and Roztoky in the central part of Bohemia, whereby two of these localities, namely Jablečno and Roztoky, also had the lowest recorded presence of the striped and the intermediate phenotype.

The counts of individuals belonging to individual colour pattern categories differed significantly between populations χ^2 (df = 14, n = 398) = 133.41, $P < 0.001$. Results of a post hoc analysis based on the residuals of Pearson’s chi-squared test for count data are summarised in Table 1. Our results confirm that localities in the north-western part of the Czech Republic (Děčín and Ústí nad Labem-Vaňov) differ significantly in the counts of both striped and spotted colour pattern phenotypes from the rest of the country (all $P < 0.001$). At these localities there is a high proportion of striped individuals. On the other hand, the Praha-Troja locality differs significantly from the rest of the country ($P < 0.001$) as there are no striped individuals. The Roztoky locality differs significantly in the count of spotted individuals

($P < 0.001$). We can thus conclude that localities in the north-western part of the Czech Republic harbour a higher proportion of the striped phenotype of *S. salamandra* than the rest of the country.

Genetic analyses

The sequencing and postprocessing of sequences resulted in a concatenated alignment of total length 1,335 bp (D-loop – 699 bp, GenBank accession numbers MZ436191-MZ436263; PDGFR*α* – 404 bp, GenBank accession numbers MZ436264-MZ436336; Rag2 – 232 bp, GenBank accession numbers MZ436337-MZ436397). We identified 11 single-nucleotide mutations in the D-loop region only one of which was parsimony-informative; the rest were singletons. Once we took the gaps into account, both the DNAsp and TCS identified eight mitochondrial haplotypes. One haplotype, represented by *S. s. longirostris* (*S. longirostris* sensu Frost 2020), turned out to be unconnected to the haplotype network because the probability of its connection to the network did not reach the 95% connection limit. One haplotype, represented by *S. s. terrestris*, was clearly separate from a group formed by the haplotype consisting of *S. s. salamandra* samples from eastern and south-eastern Europe. Then we also found a haplotype represented by *S. s. gallaica* and four haplotypes represented by individuals from the Czech Republic and Slovakia. One of these four haplotypes contained individuals from the north-western region of the Czech Republic (haplotype 2, locality Ludvíkovice), two haplotypes were represented by one individual each (both of which came from northern Moravia, namely haplotype 3, Libavá and haplotype 4, Týn nad Bečvou). All remaining individuals from the Czech Republic and Slovakia turned out to belong to the remaining fourth haplotype (haplotype 1, haplotype network in Fig. 3B).

We found only one single-nucleotide mutation in the partial PDGFR*α* sequence and two single-nucleotide mutations in the partial Rag2 sequence, but only one of those was parsimony-informative. The single-nucleotide mutation in the partial PDGFR*α* sequence was shared by the two individuals from the localities in Germany. The parsimony informative single-nucleotide mutation in Rag2 was shared by seven individuals sequenced in this study (the two individuals from Germany, four individuals from north-west of the Czech Republic, one individual from the central part of the country and one individual from the Czech-Moravian highlands) and sequence of *S. s. longirostris* downloaded from GeneBank. Both neighbour joining (Fig. 3C) and Bayesian inference-based (Fig. 3D) trees based on these concatenated data revealed a clear separation between individuals of *S. s. terrestris* and the rest of the samples studied. We therefore conclude that individuals from the Czech Republic included in our study do not belong to the *S. s. terrestris* lineage.

Discussion

We found that the striped phenotype of *S. salamandra* is most abundant in the north-western part of the Czech Republic (Fig. 2), where frequencies of the striped and intermediate phenotype differ significantly from the rest of the country (Table 1). Our finding supports and elaborates on previous reports according to which both fire salamander phenotypes are sympatric at least in a small part of the Czech Republic (Benda 2015, Moravec 2019; Fig. 1). Our results thus provoke questions regarding the historical evolutionary mechanisms which lead to the coexistence of the striped and spotted salamander phenotypes in the Czech Republic.

Different authors mention the varying distribution of the three phenotypes (striped, intermediate, and spotted) throughout Germany. The distribution of colour pattern phenotypes of *S. salamandra* in central Europe has been studied quantitatively in Germany by Klewen (1985) and in Poland by Najbar et al. (2018). In Leiberg (Germany, approximately 270 km west of the Czech Republic), most fire salamanders belong to the striped phenotype (80%), with spotted individuals making up only a small proportion of the population (< 1%) (Klewen 1985). This shows that the striped phenotype is characteristic of an area west of the Czech Republic. It has recently been reported that in eastern Upper Lusatia, the westernmost part of Poland – which is likewise adjacent to the German border (the locality is approximately 20 km from German Zittau) – there is a higher frequency of both the striped (15%) and the intermediate phenotype (57.9%) than in other Polish fire salamander populations (Najbar et al. 2018). Our data show that both the striped and the intermediate phenotype occur with the highest frequency at localities in the furthest north-west of Bohemia, Děčín (striped 53%, intermediate 40%) and Ústí nad Labem-Vaňov (striped 52%, intermediate 44%). These localities are in areas adjacent to the German border (e.g. Děčín is approximately 50 km from Zittau) in the Elbe Valley

(Fig. 2A, B). Benda (2015) showed that the striped phenotype occurs with a higher frequency (48%) than either the intermediate (22%) or the spotted form (30%) in Saxon-Bohemian Switzerland, Děčín highlands, which is similarly in the north-western part of the country, close to the German border. These results indicate that both the Czech and Polish populations of *S. salamandra* show a degree of phenotypic pattern variation comparable to that observed in the German populations, although the frequencies of the striped and spotted phenotype differ locally.

Within the Czech Republic, localities in north-western Bohemia are characterised by a high frequency of both the striped and the intermediate phenotype. In central Bohemia, we can observe a sort of hiatus, where the striped phenotype is virtually missing (< 5%) and the spotted phenotype reaches its maximum frequency (> 70%). In the eastern part of the country (Libavá), both the striped and the intermediate phenotypes are again found at slightly increased frequencies (20% and 27% respectively; compare Fig. 2A, C). This geographical trend is similar to that observed in the Polish populations, where after low occurrence in the western populations between Lusatia and Opawskie Mountains, the striped phenotype increases in frequency in the Opawskie Mountains (8%) (Najbar et al. 2018). The distance between the Czech Libavá and Polish Opawskie Mountains is less than 80 km, which means it is possible that the *Salamandra* populations of these regions are related. Our results did not, however, show a statistically significant difference between Libavá and other populations in the Czech Republic. It is thus also possible that the difference in frequency of the colour pattern phenotypes merely reflects naturally occurring random variation. Variability of colour pattern phenotypes in populations of the usually spotted *S. s. salamandra* remains virtually unknown. Future studies should attempt to quantify the abundance of colour pattern phenotypes in fire salamanders throughout their range to test the adaptive significance of colour patterns.

We found no evidence of the presence of genotypes corresponding to the *S. s. terrestris* lineage on Czech territory (Fig. 3) (for comparison between haplotypes presented in this study and haplotypes published on GenBank; Fig. S1), although *S. s. terrestris* and *S. s. salamandra* do share a contact zone in central Europe (Veith 1992, Steinfartz et al. 2000, Thiesmeier & Grossenbacher 2004, Weitere et al. 2004, Burgon 2018). Given that the contact zone between *S. s. terrestris* and *S. s. salamandra* is located at some distance from the Czech Republic any expectation of the presence of *S. s. terrestris* in the country might be unfounded. Nevertheless, estimates regarding the position of the contact zone have changed significantly over time (e.g. Freytag 1955, Gauckler 1980, Klewen 1991, reviewed in Veith 1992). Some relatively recent studies localise the contact zone only to eastern Germany or even to eastern France, Switzerland, or southern Austria (Veith 1992, Thiesmeier & Grossenbacher 2004). Weitere et al. (2004) concluded that there are two contact zones between *S. s. terrestris* and *S. s. salamandra*: one in south-western Germany, restricted to the vicinity of Karlsruhe, the other in north-western Germany, spanning several hundred kilometres from Ahaus to the Deister, near Hannover. The most recent genome-wide study suggests the presence of a lineage that shares its evolutionary history with individuals from France (i.e. individuals that would have traditionally been considered *S. s. terrestris*) in the very south of Germany and another lineage in the north, including the Elbe Valley (Burgon 2018, Supplementary Figure A4.6). Based on this high-throughput data, the putative contact zone thus would not have been limited to the north-western parts of Germany: it would extend across to the northeast. It remains unclear whether the distribution of the *S. s. terrestris* lineage reaches the very east of Germany, which may be possible given the presence of this lineage in the Elbe Valley in the north. However, at this point it must be noted that not only the distribution of central European lineages but also their subspecific status is a matter of discussion (Burgon 2018, Burgon et al. 2021).

An interesting question to be answered in the future is whether the colour pattern phenotypes are the result of shared or independent evolution of the German and Czech fire salamanders. The genetic toolkits responsible for the colour pattern of *S. s. terrestris* may be shared by genetic introgression from *S. s. terrestris* to Czech salamanders with the striped phenotype, which is a process known in other organisms (e.g. Zhang et al. 2016, Dannemann & Kelso 2017, Andrade et al. 2019). This scenario could be the case although the loci analysed in this study are not shared between the Czech fire salamanders and the *S. s. terrestris* lineage and the geographical distance between the non-admixed populations is rather long. On the other hand, the localities characteristic by an increased ratio of striped individuals are also characterised by presence of private haplotypes (Děčín – "haplotype 2", Fig. 3A, B; and Libavá and Týn nad Bečvou – haplotypes 3 and 4, Fig. 3A, B). This finding might suggest that these salamander populations have a more pronounced structure than those in the rest of the Czech Republic. It is possible that at these localities the striped phenotype evolved independently of the striped phenotype of *S. s. terrestris*. This is because 1) according to our results (Fig. 3C, D) the Czech salamanders and *S. s. terrestris* do not share any recent evolutionary history and 2) increased genetic structuring at these localities may have arisen via selection on colour pattern, as is the case in the Iberian fire salamanders (Burgon et al. 2020). The polymorphic Iberian fire salamander populations do not, however, exhibit a neutral genetic structure. This observation may imply the possibility that the Czech polymorphic populations are more ancient. However, we do not know whether any haplotype is specific for any colour pattern phenotype, because we did not examine the colour patterns of individuals that were used for genetic sampling. More detailed sampling, including reference individuals from Germany and Poland, would be needed to place our findings in a broader context of genetic variability of fire salamanders in central Europe. Moreover, different molecular techniques – such as nuclear microsatellite data or genome-wide next-generation sequencing – should be employed to improve the description of genetic variability of fire salamanders in central Europe.

Conclusions

Our data indicate that the striped phenotype of *S. salamandra* occurs more frequently in the north-western regions of the Czech Republic, which are closer to the contact zone with *S. s. terrestris*. Nevertheless, we found no evidence for the presence of genotypes corresponding to *S. s. terrestris* in Czech *Salamandra* populations. More research on the distribution of fire salamander colour patterns may reveal interesting facts about the distribution of their distinct evolutionary lineages and their evolutionary history, and perhaps also demonstrate some more general rules of evolutionary genetics.

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Supplementary online material

Fig. S1. Consensus neighbour joining tree (bootstrap-resampled, random seed = 237534, 10,000 replicates; reconstructed using Geneious 9.0.5, Biomatter Ltd.) of D-loop haplotypes, node numbers show consensus percent support, branches show nucleotide substitutions per site. 2,135 (Balkan and Ukraine individuals, red), SST1(Germany, *Salamandra salamandra terrestris*), SS1 (Czech and Slovak widespread "haplotype 1", purple), SS14 ("haplotype 2" from localities at Děčín, Czech Republic, orange), SS59 ("haplotype 3" from locality at Oderské vrchy, Czech Republic, black), SS61 ("haplotype 4" from locality at Lipník nad Bečvou, Czech Republic, yellow) represent haplotypes of samples sequenced in this study that were determined using DnaSP 5.10 (Rozas et al. 2010, Universitat de Barcelona); KY055013.1 and KY055014.1 represent haplotypes from Sudetes and Carpathians respectively in Poland determined by Konowalik et al. (2016); KT3359XX.X represent clade C haplotypes throughout of the Germany determined by Steinfartz et al. (2000); KX9519XX.X represent haplotypes from the Carpathians determined by Vörös et al. (2017). Sslongi (*S. s. longirostris*) and SSgallaica (*S. s. gallainca*) represent haplotypes of outgroup evolutionary lineages published by Vences et al. (2014).

Table S1. Tissue sampling sites and isolates identification.

Table S2. Phenotype sampling sites and colour pattern identification (1 – striped, 2 – intermediate, 3 – spotted).

(https://www.ivb.cz/wp-content/uploads/JVB-vol.-70-2-2021-Brejcha-et-al.-Fig.-S1-Tables-S1-S2-1.pdf)