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Variability in ventral spot patterns of frogs of the species *Bombina variegata* from different localities in Slovakia

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Abstract. The work is focused on the study of ventral spotting of the yellow-bellied toad (*Bombina variegata*) depending on different localities. The individuals analysed in this study come from four localities (Rosina, Žilina, Spišská Magura and Bukovské vrchy), which are quite spatially separated. The research was based on photographs of the abdominal side of individuals captured in 2013, 2018, 2020 and 2021. Evaluation of ventral spot patterns was performed using Image-Pro Plus 6.0 software. The resulting factors, determined by the statistical method of PCA (principal components analysis), were correlated with individual morphometry of individuals, sex, location of capture. Subadults had a more yellow abdomen and less variable spots. Spatial spot analysis confirmed that individual populations of frogs differed and showed differences in colouration and abdominal spots in the compared localities. Individuals from Rosina were darker, black spots were more variable in shape, their number was higher, and yellow prevailed in the B/Y ratio. Individuals from Dubeň were darker, with a strong circularity and a lower number of black spots, and a yellow colour predominated in the B/Y ratio. Individuals from Bukovské vrchy were darker, smaller in body size, with more variable and a greater quantity of black spots. Black predominated in the B/Y ratio. Individuals from Spišská Magura were lighter, with a more pronounced circularity of black spots, a high number of black spots, and black colour predominated in the B/Y ratio. We also evaluated microsatellite variation and ecomorphological differences in *B. variegata* from the locality Bukovské vrchy (Carpathian primeval beech forests, Poloniny National Park). We amplified 5 microsatellite loci, of which 4 were used to perform further fragmentation analysis. The number of alleles per locus ranged from 1 to 9. The observed heterozygosity ranged from 0 to 0.925 and expected heterozygosity from 0 to 0.846. Deviation from the Hardy-Weinberg equilibrium was not detected.

Key words: Bombina variegata, spotting - pattern mapping, biotope, locality, microsatellites

Introduction

Camouflage through background colour matching has been considered a primary force driving the evolution of colour changing ability (Kang *et al.* 2016). Animals use their distinctive colour mainly to protect themselves from predators. Bright carotene shades of yellow and fiery red draw attention to toxicity of an object. Striking patterns and colouring is often displayed during the mating season. Overall, colouration is also a result of natural selection, which takes into account several aspects of natural history, such as protection against solar radiation, thermoregulation, osmoregulation, nitrogen metabolism (e.g., Kobelt and Linsenmair 1986; Schmuek and Linsenmair 1988; Kaul and Shoemaker 1989; Tattersall *et al.* 2006). Colouration and spot pattern are important features used for traditional animal identification within taxa (Todd *et al.* 2005), distinguishing between populations (Costa *et al.* 2008), and individuals (Carafa and Biondi 2004).

Amphibians possess a diverse range of colour pattern and body markings (Hoffman and Blouin 2000). Pattern recognition is influenced by animal posture, hormonal status, injury marks, environmental influences, as well as dirt (Jørgensen and Larsen 1960; Kindermann *et al.* 2014). In anurans, it has been suggested that striking male colouration during mating time is a visual signal that supports partner recognition (Ries *et al.* 2008; Sztatecsny *et al.* 2012).

The term 'spot pattern', or 'colour pattern', refers to a mosaic of colour spots of various sizes and shapes that are arranged in a certain position relative to each other. Pattern mapping has been widely used among herpetologists in amphibian studies for several years (e.g., Donnelly *et al.* 1994; Arntzen *et al.* 2004; Moon *et al.* 2004; Davis and Grayson 2007). The principle of mapping is to study the colouration of individuals and compare spots, pattern distribution, their position, and connections. The aim of our study was to find out how spot patterns vary in different localities and populations. For this purpose, we used the analysis of photographs of the abdominal side of *B. variegata*. In the example population, we tried to evaluate the variability of microsatellites by selecting appropriate loci and summarized the findings of population studies of the genus *Bombina* based on the applicability of molecular methods in the protection of amphibians. The fire-bellied toad (*B. bombina*) and its sister species, the yellow-bellied toad (*B. variegata*), are anurans. Phylogenetically, they likely separated

during the Pleistocene glaciation (Szymura 1998). *B. bombina* is widespread throughout Central and Southern Europe (Fijarczyk *et al.* 2011), except in the southwestern part. It is distributed from central France, across central Germany, northern and western Switzerland, north-eastern Italy, Turkey (Bülbül *et al.* 2016), the Balkan region and the Carpathian Mountains (Csanády *et al.* 2020). In Europe, the yellow-bellied toad, *Bombina variegata* has an elevational range of 100 - 2100 m a.s.l. (e.g., Lác 1968; Kuzmin *et al.* 2019). In Slovakia, it is commonly located in the range of 250 to 1200 m a.s.l., or higher (Zwach 2013). *B. variegata* occurs in various water bodies, including lakes, ponds, swamps, rivers and stream pools (sometimes streams with swift currents), and springs. Its requirement for water quality is relatively low. This toad even occurs in highly polluted wetlands, where water has high concentrations of hydrogen sulphide and salts. It is the most common amphibian species inhabiting the broadest range of habitats.

Toads of the species *B. variegata* present brightly coloured yellow-and-black ventral patterns, which act as aposematic colouration (Di Cerbo and Biancardi 2010). Quantitative evaluation of ventral patterns (e.g., dark to yellow ratio, colour, amount, and shape of spots) are considered important diagnostic features for distinguishing between *B. bombina* and *B. variegata* (Lác 1961; Sas *et al.* 2005; Ghiurca and Gherghel 2007; Vörös *et al.* 2007; Covaciu-Marcov *et al.* 2009), but may also be important features within individual populations or individual characteristics (Delarze *et al.* 2000; Seidel *et al.* 2001). The method of analysing digital images through graphics programs is a relatively inexpensive and non-invasive, albeit time-consuming process undertaken by individuals (Arntzen *et al.* 2004; Patel and Das 2020). This method is suitable for amphibian species that have highly variable dorsal or ventral spot patterns. The advantages of spot mapping include its non-invasive nature, re-use of images, and feasibility in the field in a relatively short time. This way, on an individual basis, we can create a history of photos that allow a comprehensive examination of population size (Plăiașu *et al.* 2005).

The use of molecular methods in population studies allow not only knowledge of genetic diversity within a population, but also knowledge of the status of species and populations over relatively short periods of time in contrast to demographic studies, which can last up to several years (Pechmann *et al.* 1991). Suitable methods for evaluating the genomes of amphibian populations are analysis of highly variable genetic markers such as microsatellites, SNP markers, or the AFLP method (Storfer *et al.* 2009). Hypervariable genetic markers, such as microsatellites or SNP markers, are widely used in landscape genetics, mainly because of their high statistical ability to distinguish the genetic diversity of individuals, groups, or populations (Storfer *et al.* 2007). Another method useful in amphibian protection is real-time PCR (a method that is a variation of the standard polymerase chain reaction). Using it, we can visualize SNP markers needed to characterize population and species diversity. Microsatellites have been shown to be particularly useful for measuring gene flow and migration, for classifying individuals in the most likely population of their origin, for measuring effective population size by comparing the frequency of alleles between generations, and for detecting demographic bottlenecks in the past (Storfer *et al.* 2009).

Microsatellites represent the repetitive tandem stretches of DNA that occur in the genomes of prokaryotic and eukaryotic organisms. Within the genome, they are in regions encoding proteins but also in non-coding regions (Tóth *et al.* 2000). The most common are repeats of 1 - 6 base pairs with a length of 5 - 40 repeats (Selkoe and Toonen 2006). Both ends of microsatellites are bounded by adjacent regions of DNA called flanking regions. The sequences of these regions are generally identical between individuals of the same species (sometimes within related species) and based on them we can characterize specific microsatellite locus. Therefore, locus-specific primers are proposed that allow us to amplify microsatellites in a PCR process (Selkoe and Toonen 2006).

In population genetics studies, microsatellites have the potential to provide up-to-date information and estimates of migratory routes, to distinguish between migration and panmixia and can also reliably determine the relationship of individuals (Selkoe and Toonen 2006). Due to its high mutation rate, it is one of the most informative molecular markers (Hoshino *et al.* 2012). Research on microsatellites in the genus *Bombina* focuses mainly on the study of population structure, gene flow between populations, migration, and hybrid zones.

Material and Methods

Sampling

Research took place in mountainous areas of northern Slovakia in 2013, 2018, 2020, and 2021. Sample collection was conducted in Bukovské vrchy mountain (Poloniny NP; N 49.035527°; E 22.327672°) in 2013, within pools on or near forest roads in the shade of a beech forest. The second locality was the cadastre of the village Rosina in 2018; a marginal part of the forest with a temporal pond (N 49.179959°, E 18.75314°). In 2020, two sites in Spišska Magura - Lendak southwest (N 49.25590°, E 20.35353°), and Príslop northwest (N 49.2815°, E 20.226°) were chosen, with pools on forest roads. Lastly, in 2021, temporary pools in the Dubeň forest park (Žilina, N 49.234274°, E 18.753079°) were used as a locality.

The animals were caught using hand nets or directly by hands. They were measured (head width, body length, length of leg, thigh length) and weighed immediately in the field (Fig. 1). Gender was determined based on external morphological features, like horny mating calluses (nuptial) on the forearm and fingers of the forelegs and sometimes on the hind limb in males (Mikuliček and Vongrej 2005). Females were identified at the time of mating by a wider abdomen, indicating egg maturation. Individuals who did not show these signs of sexual activity were marked as subadults.

Photographs of the ventral side of each individual (Fig. 2) were taken for ventral spot analysis, with an emphasis on fixation, so that it was possible to

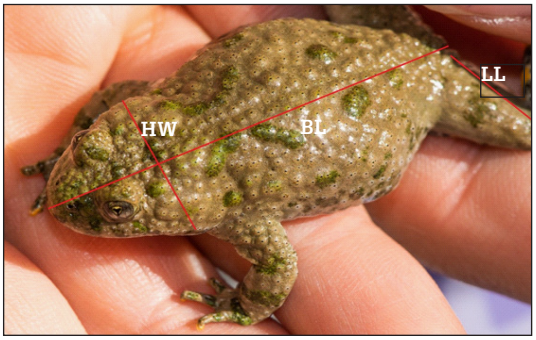

Fig. 1. Measurement lines. BL - body length; HW - head width; LL - leg length (Photo: V. Ruček, 2020).

Fig. 2. Taking a picture of the ventral side of *B. variegata* in the field (Photo: A. Zakharova, 2020).

scan the largest possible area without deformations caused by gripping the frog. A tissue sample was taken from the captured individuals (by cutting a piece from the hind limb finger) for DNA extraction and subsequent analysis of microsatellites. Immediately after handling, the animals were released at the capture site.

Spot analysis

The method of quantitative pattern analysis (applied to *B. variegata* and *B. bombina*) is described by Vörös *et al.* (2007). Abdominal spot analysis was performed using the highly specific Image-Pro Plus 6.0 program (2D Image Analysis Software by Media cybernetics Inc.; IPro). The preparation of the photos went through several stages of editing to achieve acceptable results. In the first step, the image was corrected using Microsoft Paint (Microsoft Windows). In this step, the alignment and cropping of the image to the format required for our purposes was achieved, followed by colour adjustment for simplified manipulation in IPro, so that different shades did not interfere with the subsequent immediate analysis of the finished image. The most extensive work in Paint was hand-drawing areas (black spots) with their tannic unification for further manipulation. After this stage, the image could continue to be edited in IPro: adjusting the colour spectrum of the examined sample as well as pigment designation (designation of the specific colour of pixels - in fact it is black but passing through the adjustment filters it is white, which is referred to as a preoccupation of interest; Fig. 3). Thus, all pixels of the same colour were marked and combined into the examined spots exactly according to the manual plot of the flood spot. The program automatically extracts the necessary data from which a matrix is created for further evaluation and analysis.

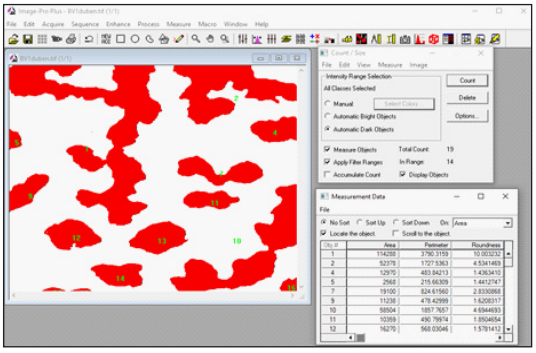

Fig. 3. Spot analysis. Preview from IPro (Photo: A. Zakharova, 2021).

Basic data were selected in IPro, including object area (total area of black spots; does not include holes), perimeter (per sum – perimeter of all black spots; per mean – average perimeter of black spots), number of black spots, and mean roundness (circularity).

From these values, other variables characterizing the abdominal pattern were calculated: per cent of black colour (ratio of total black spot area to total calculated abdominal area), per cent of yellow colour (expressed as the difference between total abdominal area and per cent of black colour), B/Y (ratio colour to yellow), RMPA (ratio of average black spot content to average black spot circumference).

DNA isolation and PCR

DNA was isolated from tissue samples using the commercially available DNeasy Blood and Tissue Kit (QIAGEN, USA). Isolation was performed using a standard protocol (Purification of Total DNA from Animal Tissues).

The isolated DNA samples were subsequently analysed in 1 % agarose gels in 1 x TBE buffer. For DNA visualization, ethidium bromide was added to the gels at a final concentration of 0.5 $\mu g.ml^{-1}$. Electrophoresis was performed for approximately 1 hour at a potential gradient of 8 - 10 $V.cm^{-1}$. We observed DNA fragments under UV light. The size of the fragments was determined based on their mobility in agarose gels by comparison with a 1 kb ladder.

For testing microsatellites, we used loci amplified in *B. variegata* by Stuckas and Tiedemann (2006), and Hauswaldt *et al.* (2007). The individual loci that were amplified using the polymerase chain reaction (PCR) are listed in Table 1.

The reaction mixture contained the following reagents: ultrapure water, 1 x GoTag Flexi Buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.2 mM forward primer, 0.2 mM reverse primer, FirePol polymerase, DNA sample. In the case of locus 5F, we increased the concentration of $MgCl_2$ from 1.5 to 2.0 mM. The samples were then placed in a thermal cycler with a pre-set program designed according by Stuckas and Tiedemann (2006). As the reaction produced non-specific products, it was necessary to optimize the conditions of the PCR reaction - they increased annealing

Lokus	Forward and reverse primer	T_a	References
Bobom B13	For: 5´ -Cy5-ATATTTCTTGCTATGTTGATG-3´ Rev: 5´ -AATTGTTTAACTTATTTTATA-3´	46 °C	Stuckas and Tiedemann (2006)
Bobom F22	For: 5´ -Cy5-AGGCAAAGGATTCTGAGAATG-3´ Rev: 5´ -CCTTCAAAGTCGAAAAATATT-3´	56 °C	Stuckas and Tiedemann (2006)
Bobom F2	For: 5´ -Cy5-AGCAGAGATGAGAGGACAGTG-3´ Rev: 5´ -TCAGGGGTAGCAGATTTTCA-3´	60 °C	Stuckas and Tiedemann (2006)
Bobom F5	For: 5´ -Cy5-ATGAATTGGAAGGTAAGAACTTACACC-3´ Rev: 5´ -CAAATGATACAAATCAAGTGGAATGG-3´	63 °C	Hauswaldt *et al.* (2007)
Bobom 1A	For: 5´ -Cy5-ATGTGGCTTCCATTGACCTTTGC-3´ Rev: 5´ -CATGCCAAGAAGGATTGAGTCTGT-3´	65 °C	Hauswaldt *et al.* (2007)

Table 1. List of primers used (forward primers fluorescently labelled with Cy-5, T_a = annealing temperature).

Initial denaturation	95 °C 2 min.	
Denaturation	95 °C 1 min.	
Annealing	Primers F2, F22: (T_a + 3) °C 1 min. Primers B13, 1A, 5F: (T_a + 5) °C 1 min.	3 cycles
Extension	72 °C 1 min.	
Denaturation	95 °C 1 min.	
Annealing	Primers F2, F22: (T_a + 3) °C 1 min. Primers B13, 1A, 5F: (T_a + 5) °C 1 min.	38 cycles
Extension	72 °C 1 min.	
Final extension	72 °C 40 min.	

Table 2. Temperature and time profile.

temperature. The resulting temperature and time profile is shown in Table 2. After PCR, the products were visualized using electrophoresis on a 1 % agarose gel using a 50 bp DNA Step Ladder as a weight standard.

Analysis of microsatellites

Analysis of PCR products was conducted using fragment analysis performed in the GenomeLab GEXP sequencer (Beckman Coulter), which is based on the principle of capillary electrophoresis and detection of fluorescently labelled fragments by laser. One sample contained 0.16 µl of PCR product, 29.5 µl of Sample Loading Solution (SLS) and 0.16 µl of DNA Size Standard-400. Primary data obtained by fragment analysis were evaluated using the GenomeLab-Fragment Analysis software program (Beckman Coulter), which is part of the sequencer. The output data from this program were graphs showing individual alleles. For each individual, we subtracted the size of the amplified alleles from the graph, and used it in statistical analysis in Cervus 3.0.6 (Kalinowski *et al.* 2007) and in the construction of a phylogenetic tree. The phylogenetic tree was constructed using the distance matrix by the UPGMA method (unweighted pair group method analysis). Jaccard's distance was used for the distance matrix. The following characteristics of genetic diversity and population differentiation were also calculated in Cervus: expected (HEXP) and observed (HOBS) heterozygosity, polymorphic information content (PIC), Hardy-Weinberg equilibrium test, allele frequency, zero allele frequency, number of alleles per locus, and number of alleles. Using Genepop 4.2.2 (Rousset 2008), we calculated the FIS inbreeding coefficient.

Statistical analysis

Statistical evaluation of ventral spotting was performed in Statistica Ver. 8 (StatSoft, USA). To determine the main component weights and percentage of variation among abdominal spot patterns, we used Principal component analysis (PCA). We used One-way ANOVA and Tukey's test HSD (honestly significant difference) at a 95 % confidence level ($p < 0.05$) to evaluate the impact of variables (individual predispositions, locality) and main factors.

Results

Principal components method

A total of 196 individuals of *B. variegata* were captured and documented (Rosina n = 47, Spišská Magura n = 29, Dubeň n = 20, Bukovské vrchy n = 100).

Using PCA, we have identified seven main factors (F) with the highest variation (Table 3): F1 - melanism, F2 - image processing errors; F3 - animal size (head width, body length, weigh), F4 - circularity, F5 - leg length, F6 - number of dark spots, F7 - B/Y ratio. The PCA method shows that morphometric variables, in their variability, behave independently of variants that resemble melanism and spotting. Factor 2 defines irregularities in the processing of the photograph, and it is defined mainly by the total abdominal area. This characterizes the entire analysed section of the photograph, followed by the perimeter sum, and the total area of a black spot. Differences in the quality of photographs are

Variable	Factor 1	Factor 2	Factor 3	Factor 4	Factor 5	Factor 6	Factor 7	Factor 8
Body length	-0.29	-0,51	**0.67**	0.18	0.08	-0.06	-0.06	0.04
Head width	-0.20	-0,48	**0.75**	0.06	-0.13	-0.06	0.03	**-0.29**
Hind limb /femur	0.41	-0.00	0.35	-0.33	**-0.69**	-0.31	-0.04	0.04
Weight	-0.26	-0.45	**0.72**	0.11	0.14	0.23	0.05	**0.25**
Total abdominal area	-0.49	**0.76**	0.32	0.01	0.13	-0.05	0.00	-0.13
Total area of black spots	**-0.76**	**0.58**	0.15	0.13	-0.09	-0.09	-0.01	0.07
Average area of black spot	**-0.91**	0.17	0.03	-0.20	-0.04	-0.08	-0.20	0.15
Black colour %	**-0.71**	-0.37	-0.37	0.29	-0.32	0.00	0.14	0.03
Yellow colour %	**0.71**	0.38	0.37	-0.29	0.32	0.00	-0.15	-0.03
Ratio B/Y	-0.65	-0.50	-0.33	0.16	0.01	0.08	**-0.34**	-0.18
Number of dark patches	0.57	0.42	0.14	0.26	-0.41	**0.41**	-0.23	0.06
Mean roundness (circularity)	-0.58	-0.36	-0.09	**-0.64**	-0.11	0.29	0.04	0.00
Perimeter mean	**-0.89**	0.04	0.01	-0.38	0.05	0.08	-0.01	-0.03
Perimeter sum	-0.54	**0.68**	0.22	0.08	-0.17	0.29	0.18	-0.12
RMPA	**-0.86**	0.35	0.05	0.16	0.05	-0.25	-0.07	0.08
Variance %	**39.7**	**20.5**	**14.9**	**7.1**	**6.5**	**3.9**	**2**	**1.7**

Table 3. Factor coordinates of variables according to correlation matrix in PCA was used. The highest correlations are in bold.

caused by optical properties of the lens, sensitivity of the sensor in poor lighting conditions, ambient light conditions, image crop range, or positioning of the individual sample out of plane of sharpness. The quality of the image depends on the camera, the photographer, and both automatic and manual post-processing.

Influence of age and gender on the pattern of ventral spots

Factor 1 presents the degree of dark colouration of the abdominal side (total abdominal area, total area of black spots, perimeter sum, RMPA - ratio of black spot area to spot circumference length), represents the degree of melanism. The abdominal spot pattern in subadults is significantly lighter (fewer dark spots) and with less variable black spots ($F(2,179) = 9.96$, $p = 0.0008$) than in adults (males, females) (Fig. 4).

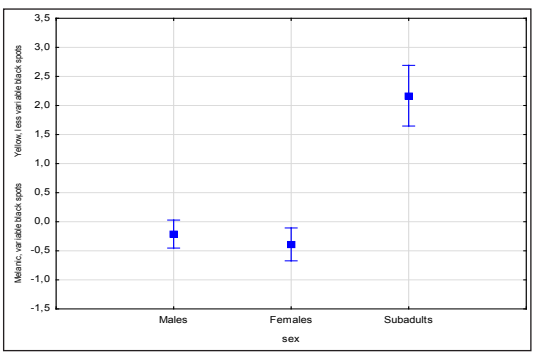

Fig. 4. Factor coordinates of variables at the Factor 1. Subadults show significantly lighter abdominal discolouration and lower variability of dark spots as males and females. Data in factor coordinates are given in averages and SD - standard deviation (±).

Factor 3 represents size variability and clearly determines the dependence of individual size (body length, head width, weight) on age status (subadults, adult males and females) adults are significantly larger ($F(2.179) = 5.69$, $p = 0.004$). Factor 4 is related to the circularity of the spots but is independent of age and sex ($F(2,179) = 2.33$, $p = 0.09$). Factor 5 takes into account the length of the hind leg (femur). Subadults have significantly longer legs than adult males and females ($F(2,179) = 8.90$, $p = 0.0002$). The number of dark spots (Factor 6) is not significantly determined by age and sex ($F(2,179) = 0.26$, $p = 0.77$). Factor 7 is the ratio of black to yellow within the spot pattern. This ratio is also not statistically significantly conditioned by age and gender ($F(2,179) = 0.17$, $p = 0.85$).

Spatial variation in the pattern of ventral spots and local population characteristics parameters

In the spatial analysis of abdominal spotting, we excluded a group of subadults, due to significantly different types of spots. Thus, when comparing spatial variability, we focused only on the sexually mature stages.

The darker colouration of the abdominal patterns characterized by Factor 1 significantly differentiates the population of frogs from the Spišská Magura locality (Fig. 5). Individuals of other localities did not differ from each other ($F(3,180) = 14.06$, $p = 0.00000$). In localities with more melanic colouration of frogs (Rosina, Dubeň, Bukovské vrchy), they have a larger average area of black spots and at the same time a larger circumference (total perimeter) and RMPA, as irregular curvature of spots also increases their circumference.

We also evaluated the differences between localities on the basis of Factor 3, which is directly related to the morphometry of individuals (Fig. 6). It was found that in the locality Bukovske vrchy individuals were significantly smaller than in other localities ($F(3.180) = 12.479$, $p = 0.00000$).

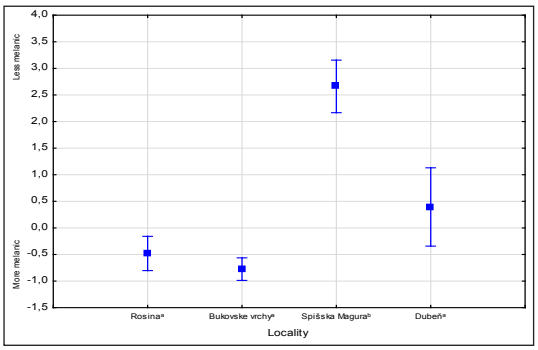

Fig. 5. The population from Spišská Magura differed significantly between the populations of individual localities (index b). Negative values indicate individuals with greater variability and areas of black spots, positive values indicate individuals with a lighter (more yellow) abdomen and less variability of black spots.

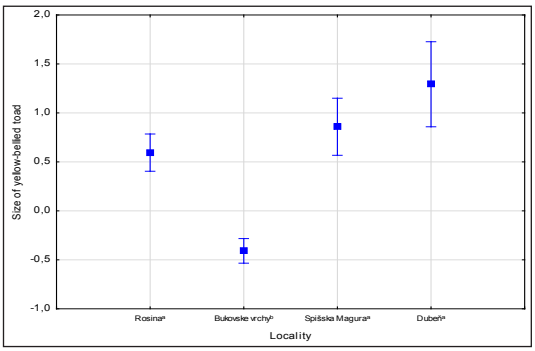

Fig. 6. Differences in the size of individuals in localities. Individuals in the population of frogs from Bukovské vrchy is significantly different (index b) from other localities. A higher numerical value presents larger individuals.

Also, the shape of the spots defined by Factor 4 (roundness) varies between sites (Fig. 7). Individuals from the Spišská Magura and Dubeň localities had more rounded spots, (i.e., they showed less variability of spot curvature ($F = (3,200) = 9.6300$, $p = 0.0001$)).

The number of dark spots (Factor 6) was significantly lower in individuals from the Dubeň locality ($F (3,200) = 7.42$, $p = 0.0001$). The number of dark spots was higher in Rosina, Spišská Magura and Bukovské vrchy (Fig. 8).

The ratio of black and yellow (individual pattern) on the abdomen of *B. variegata* (Factor 7) also exemplifies significant differences between localities ($F (3,200) = 5.36$, $p = 0.001$). The most similar patterns occurred in Bukovské vrchy and Spišská Magura; these individuals have a larger area of dark spots and a higher number of dark spots. Populations from Rosina and Dubeň show lighter colouration (more yellow) and fewer black spots (Fig. 9).

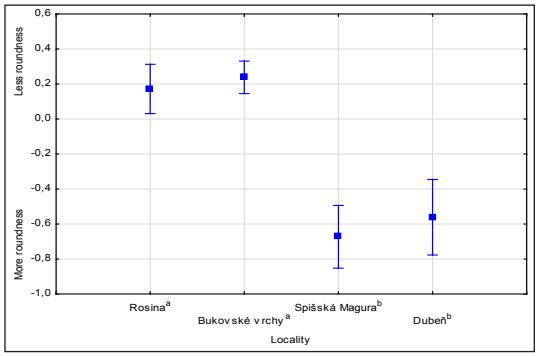

Fig. 7. Rounding of spots in individual localities. While in Rosina and Bukovksé vrchy individuals have more variable curvature of spots (index a), in Spišská Magura and Dubna the spots showed higher circularity (index b).

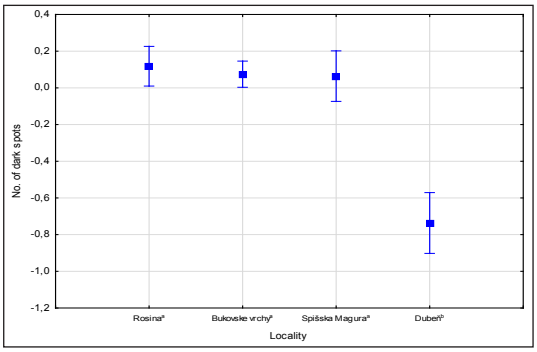

Fig. 8. Dubeň (index b) manifests as a locality with individuals, who have a smaller number of dark spots than other localities (index a).

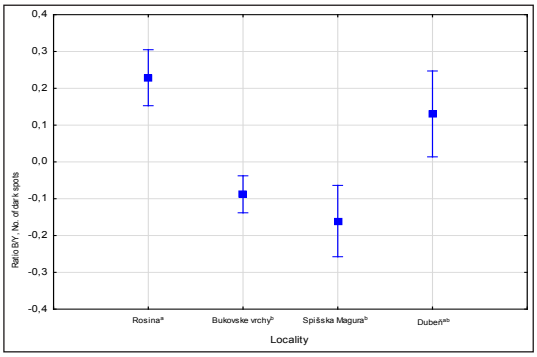

Fig. 9. Abdominal spot pattern conditioned by B/Y ratio and a number of dark spots in localities. Negative values describe individuals with a larger area and a higher number of dark spots), positive values represent individuals with a lighter colour (more yellow) and a smaller number of black spots. Between the localities of Bukovské vrchy and Spišská Magura (index b), frogs did not differ. Individuals from the locality of Dubeň (index a, b) do not differ from any compared group.

Genetic analysis

DNA was isolated from the samples taken in the field and this was subsequently used in analysis of microsatellites. After optimization of the PCR conditions, the individual loci were amplified.

The resulting PCR products (except for the Bob F2 locus, for which the standard was not large enough) were analysed in the sequencer by fragment analysis. From the output graphs, we read the sizes of the amplified alleles for each individual, and in the CERVUS 3.0.6 program, we calculated indicators of genetic diversity. In addition to the monomorphic locus Bob F22, the remaining analysed loci were polymorphic.

The number of alleles per locus ranged from 1 to 9. The lowest number of alleles per locus was recorded at the locus by Bob B13, and the highest at Bob by 5F. No significant deviation from the Hardy-Weinberg equilibrium was found for the Bob B13 and Bob 1A loci. However, for the Bob F22 and Bob 5F loci, this test was not performed due to the low amount of data. The observed heterozygosity ranged from 0 to 0.92 and expected heterozygosity in the range of 0 to 0.846 (Table 4). A comparison of the observed and expected heterozygosity confirmed the trend toward lower observed heterozygosity at the Bob B13 locus, which also had a higher value of the FIS inbreeding coefficient. The values of the frequency of zero alleles for individual loci are close to zero, and thus indicate the absence of zero alleles in the population. Detailed values of the basic characteristics of genetic diversity for individual alleles are shown in Table 5.

The phylogenetic tree constructed using the distance matrix by the UPGMA (unweighted pair group method) is shown in Fig. 10. It can be seen from the figure that samples 54 and 62, and samples 47 and 61 show identical individuals. This phenomenon is due to the small number of loci on which individuals have the same allele sizes. Therefore it was not possible to distinguish them from each other. In addition, the monomorphic locus Bob F22 does not give any information for comparison, as it does not change. It appears to be a locus that does not undergo mutations over time. We can also see from the tree that the group of individuals 19, 34, 37, 42, 46, 74, 98, 102 is separated from the others. This is not due to geographical separation of these individuals from others (for example, by the influence of geographical barrier), as these are individuals from different habitats. It is most likely that this discrepancy is due to the low number of loci used for analysis.

Locus	H_{OBS}	H_{EXP}	P_{IC}	HW	F (Null)	N_A	F_{IS}
Bobom B13	0.450	0.604	0.521	NS	0.1267	3	0.2571
Bobom F22	0.000	0,000	0.000	ND	ND	1	-
Bobom 5F	0.925	0.846	0.816	ND	-0.0521	9	-0.0882
Bobom 1A	0.675	0.648	0.586	NS	-0.0295	7	-0.0335
Primer	0.51225	0.5245	0.4806	-	0.0150	5	0.0313

Table 4. Overview of genetic diversity characteristics for specific loci. HOBS = observed heterozygosity, HEXP = expected heterozygosity, PIC = polymorphic information content, HW = deviation from Hardy-Weinberg equilibrium (NS = not significant, ND = not done), F (Null) = frequency of zero alleles, NA = number of alleles per locus, FIS = inbreeding coefficient.

Locus	Allela	Frequency	Homs.	Hets.	Frequency	Frek NULL
Bobom B13	113	42	18	12	0.5250	0.4881
	119	26	10	8	0.3250	0.2535
	121	12	8	2	0.1500	0.1317
Bobom F22	135	80	0	40	1	ND
Bobom 5F	115	6	6	0	0.0750	0.0778
	119	17	17	0	0.2125	0.2410
	123	20	16	2	0.2500	0.2576
	127	6	6	0	0.0750	0.0778
	131	10	10	0	0.1250	0.1336
	135	3	3	0	0.0375	0.0381
	139	2	2	0	0.0250	0.0253
	143	11	11	1	0.1625	0.1629
	147	3	3	0	0.0375	0.0381
Bobom 1A	322	41	21	10	0.5125	0.5249
	326	23	19	2	0.2875	0.3105
	330	1	1	0	0.0125	0.0126
	341	2	2	0	0.0250	0.0253
	345	1	1	0	0.0125	0.0126
	349	9	7	1	0.1125	0.1055
	353	3	3	0	0.0375	0.0382

Table 5. Overview of genetic diversity characteristics for individual alleles: Frequency = number of allele occurrences in the genotype set, Hets = number of heterozygotes for a given allele in the genotype, Homs = number of homozygotes for a given allele in the genotype, Frequency = number of allele occurrences divided by the total number of alleles, FrekNULL = allele frequency taking into account the possible presence of null alleles.

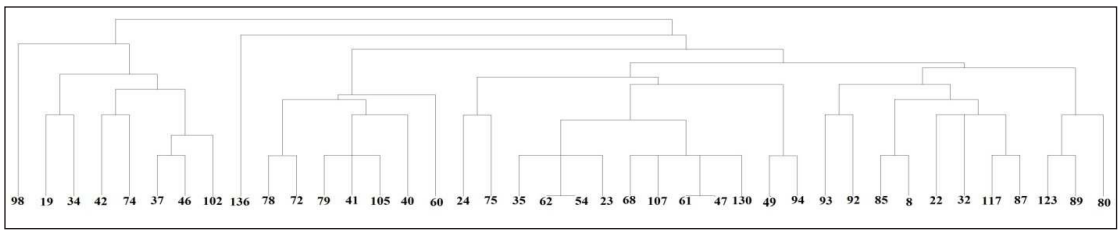

Fig. 10. Phylogenetic tree compiled by the UPGMA method.

Discussion

The study of the belly colour pattern of *Bombina* species and the identification of interspecific hybrids has been of interest for more than a century. Several useful methods have been developed to analyse the abdominal patterns of two species based on similar symbols: distribution, position, size, and colour of light spots. However, without precise quantification, these features appear subjective. An example of a practical application is the results of Budapest scientists, Vörös *et al.* (2007), who showed that *B. variegata* has a smaller quantity of larger, and more regular spots and that the colour (RGB) composition of this species consists of higher red and green components, indicating a bright yellow colour. Conversely, *B. bombina* has darker, orange spots, with lower values for the red and green colour components. The results also showed that using their computer method combined with multivariate statistical analysis, it was possible to distinguish "pure" populations of *B. bombina* and *B. variegata* based on a ventral colour pattern. Features derived from the ventral colour pattern were useful for separation, except for the least variable blue component of the colour characteristics.

Analysis of individual spot patterns in *B. variegata* is a tool to better understand the degree of variation between individuals of a particular population. Individual identification is vital to revealing demographic patterns to understand population dynamics (Patel and Das 2020), and to improve species protection. Photo identification is a non-invasive technique, which is a clear advantage (Renet *et al.* 2019). Digital evaluation of spot patterns thus becomes an inexpensive and particularly non-invasive technique that can be used in studies of amphibian populations that do not undergo significant changes in spot patterns in adulthood. Yellow-bellied toads are a suitable model for studying environmental influences, due to their common availability in the territory. Their colouration and spot pattern stabilize following their first year (Novitsky *et al.* 2001), and they tolerate short-term manipulation very well. Barandun and Reyer (1997) report that mating was observed in individuals within one hour after handling.

Quantitative characteristics associated with the pattern on the ventral side of the body, such as the ratio of black to light areas, number, shape and relative position of spots, are considered by many authors to be the main diagnostic features distinguishing *B. bombina, B. variegata* and their hybrids (Lác 1961; Gollmann 1984; Plăiașu *et al.* 2005; Sas *et al.* 2005; Vörös *et al.* 2007; Covaciu-Marcov *et al.* 2009). The advantage of the abdominal spots of *Bombina* individuals is that the pattern is permanent after metamorphosis (Streich *et al.* 1997; Gollmann and Gollmann 2012). Most changes in the pattern occur a few weeks after metamorphosis, and therefore it is not recommended to use photographs of the abdominal part taken during the period when the complete pattern has not yet been created (Gollmann and Gollmann 2011). The development of black spots, which are a characteristic individual feature of *B. variegata*, is also related to the ontogenetic development of colouration. Factor 1 represents variations in colour and spotting. In correlation with age, it consistently evaluates subadult individuals as lighter with less variability of black spots. However, the circularity of spots (F 4), the number of dark spots (F 6) and the ratio of B/Y colour (F 7) is not significantly conditioned by age. Thus, we can say that despite significant differences in colouration, individual differences in basic abdominal spot patterns are subject to ontogenetic development to a limited extent. This phenomenon is partly confirmed by previous studies, where individual patterns of spotting and discolouration stabilize only after the first wintering (Novitsky *et al.* 2001). Older animals are more characteristic of darker colours in the process of ontogenesis (Kraus and Allison 2009). It is also thought that changes in variations in colouration are hormonally conditioned during ontogenesis (Richards 1982; Hayes and Menendez 1999; Hoffman and Blouin 2000).

Juveniles commonly use cryptic dyes and mature into a striking colouration in adulthood (Bulbert *et al.* 2018). Thus, the cryptic colouration (camouflage) changes with age. This defence mechanism is used by organisms to mask their appearance, usually to blend in with their surroundings. Temporary pools, especially on forest or field paths, often have a cloudy sandy appearance. The light colour of subadult forms could be camouflage in this case. Younger individuals are also exposed to more frequent predator attacks due to their insufficient experience and lower mobility. Predators can be detrimental to *B. variegata*, and Barandun and Reyer (1997) mention that this predation is extremely dangerous in the early stages of life. The opposite defensive strategy is an aposematism, which is a striking warning colouration by which the animal warns predators that it is poisonous. The yellow colour generally discourages animals and sends a warning signal against the attack and possible poisoning. The yellow-bellied toad displays a cryptic dorsal colouration in different shades of grey and brown and it's aposematic colouring is a conspicuous ventral yellow with contrasting black spots (Kwet 2015). Bright colours can warn predators that potential prey is toxic, thus,

we can infer that predators are the most significant contributing factor to the development of aposematism (Rojas *et al.* 2020). However, the study of colour polymorphism in frog venom suggests instead this is the result of parental repression and sexual selection (Deegan and Engel 2019), as evidenced by a study by Jeckel *et al.* (2019) which found that *Adelphobates galactonotus* (Dendrobatidae) from two sites with different colour morphs did not differ in their toxin profile.

Amphibians often change colour during development, responding to various environmental factors. This colour variability and different melanin content have been shown to play an important role in thermoregulation (Tattersall *et al.* 2006), reproduction, protection against UV radiation (Garcia *et al.* 2004) and predation (Rudh and Qvarnström 2013). Licht and Grant (1997) hypothesized that the depleting ozone layer and increasing amount of UV-B radiation may cause increased amphibian mortality. Effective barriers to the propagation of UV-B radiation are the depth of water and its colouration (Smith and Baker 1979; Scully and Lean 1994). This is consistent with our observations that individuals have a higher black pigment content in cleaner, non-turbid water, which should transmit UV-B radiation better.

However, this hypothesis required additional research as conflicting results have characterised this area of study to-date. In some species, higher mortality was observed after exposure to UV-B radiation, but in others there was no effect (Licht and Grant 1997; Rudh and Qvarnström 2013). The advantage of darker pigmented individuals is their ability to reach a higher body temperature faster than ambient temperature (Brattstrom 1963; Duellmann and Trueb 1994; Vences *et al.* 2002), which in turn allows them to exhibit higher activity and more rapid growth (Lillywhite *et al.* 1973). Lighter colouring, on the other hand, prevents the body from overheating, but may increase the risk of being detected by predators (Tattersall *et al.* 2006). Our results showed that frogs found in muddy water were lighter, which may indicate that they do not need significant protection against UV-B radiation in the form of a higher melanin content, and that the predominance of a strong yellow colour may not be a disadvantage for them in avoiding predation. Thus, the observed difference in the level of pigmentation may be due to several factors. According to Stugren and Vancea (1968), the ratios between yellow and black, in *B. variegata* should not be considered as the result of metabolic changes influenced by the origin of the environment alone. While these different forms can also be caused by more random mutations, their frequency and distribution indicate that they are below environmental control. At present, many causes of these colour adaptations remain undetected and unexplored, while few genes have been discovered that affect the pigmentation of individuals (Rudh and Qvarnström 2013).

Variation in the pattern of ventral spots according to spatial distribution

The yellow-bellied toad belongs to a species that engages in daily activity, is less bound to water, and possesses migratory ability. It reproduces in smaller stagnant waters and periodic pools on mountain pastures, forest road tracks, flooded pits, etc. Some studies suggest that *B. variegata* prefers free wastewater. According to the research of Csanády *et al.* (2020), one of the most important factors positively influencing the fauna of *B. variegata* in temporary rainwater pools, is the volume and area of the rain pools, while the presence of shadows has a negative effect. The influence of other parameters (e.g., water temperature, biotope structure, the presence of chemical compounds or chemical properties, pH) was also significant (Csanády *et al.* 2020). Temporary lakes or pools on field roads are characterized by low or zero vegetation cover and high solar radiation. Research by Warren and Buttner (2008) also confirms that the probability of occurrence of the *B. variegata* increased in water reservoirs on bare land (rare vegetation), as a result of human activity.

In our research, we analysed ventral spotting in four populations of *B. variegata* from different areas of Slovakia. The sites were spatially distant to ensure a reduction in the genetic impact in colouration and spotting. Two localities were from the vicinity of Žilina (Rosina, Dubeň), one from Spišská Magura and one from Bukovské vrchy. All areas were large, and sampling (individuals) was not limited to a small area where all individuals could be assumed to belong to the same family line. Spatial spot analysis confirmed that individual populations of frogs differed and showed differences in colouration and abdominal spots in the compared localities. Individuals from Rosina were darker (F 1), with black spots that were more variable in shape (F 4), higher in quantity (F 6), and with a prevalence of yellow in the B/Y (F 7). Individuals from Dubeň were darker (F 1), with a strong circularity (F 4), lower number of black spots (F 6), and a yellow colour predominance in the B/Y ratio (F 7). Individuals from Bukovské vrchy were darker (F 1), smaller in body size, with a greater quantity and greater variation of black spots (F 4, F 6), and a predominance of black colouration in the B/Y ratio. Individuals from Spišská Magura were brighter (F 1), with a more pronounced circularity and higher number of black spots (F 4, F 6) and a predominance of black colouration in the B/Y ratio.

Environmental conditions such as brightness, structuring of habitats, and presence or absence of vegetation have a strong impact on the conspicuousness, and thus on the transmission and detectability of, a visual signal (Endler 1992; Peters 2008). The locations in our research were diverse. In the case of Rosina, the habitat was represented by small temporary tanks, shaded by the forest or buildings. In both areas, the water was muddy and exposed to the sun only a limited time period. The Dubeň locality is characterized as a forest road sufficiently open to sunlight, but interspersed with sufficient tree-cover to provide shade; there is an abundance of grassland, and the water is muddy. Spišská Magura is characterized by temporary water pools formed on the forest road. All collection points were in the open out of the shade. In the locality of Bukovské vrchy, there are natural as well as artificially created water areas, reservoirs, and areas exposed to sunlight (Karaščáková 2014). Due to the characteristics of these sampling sites, it follows that the darker coloured individuals exhibited by Factor 1 occur in localities with lower solar ra-

diation. On the contrary, in the explicitly sunny site (Spišská Magura), the individuals were characterized by lighter colouration. The colour of the water may have an additional influence on this factor. In Spišská Magura, the water was quite turbid and muddy with a light brown to yellow colour, unlike other locations. We can assume that this represents an adaptation to the environment in which each frog develops.

For anurans, short-term adaptation to the environment is common. Short-term adaptation of *B. variegata* to environmental conditions was also confirmed in a study by Preißler *et al.* (2021), wherein frogs transferred to a lighter substrate were observed to lighten in colour in just one day, whereas frogs transferred to a darker substrate steadily decreased in brightness. However, it should be noted that the above study focussed on dorsal discolouration, while our study focusses on patterns of abdominal spots. Many amphibians get darker against a black background, or lighter against a white background, owing to the dispersal of melanin-containing organelles (melanosomes) or aggregation of iridophores (cells with platelets involved in structural colouration) (Preißler *et al.* 2021). Geographical variations in aposematism and crypsis (Mappes *et al.* 2005) are also common. Thus, we can assume that the amount of sunlight and the surrounding vegetation plays an important role in the formation of abdominal spot patterns and the overall colouration of *B. variegata*. The colouration of a particular population group may be the result of the collective evolution of colouration and toxicity (Summers and Clough 2001; Summers *et al.* 2003).

In general, it is not possible to determine which of the environmental factors has the greatest effect on the spot pattern. Environmental conditions also include the presence or absence of predators. The variety of colour patterns seen in animals tends to reflect different defensive strategies (Ruxton *et al.* 2004). In this respect, the colour and spot patterns are important. However, it is not only short-term predisposition to adapt to the environment that protects animals from negative effects of the environment. For example, ontogenetic colour change and non-reversible change in body colour occur as individuals transition between different life stages (Booth 1990; Grant 2007; Wilson *et al.* 2007). Therefore, unique stain patterns are often subject to more long-term development.

Environmental factors that affect anuran colour changes include the intensity of light, background colour, and temperature (Kang *et al.* 2016). In general, frogs are more brightly coloured in stronger light and higher temperatures (Stegen *et al.* 2004). The darkening of animals with age or ontogenesis is largely expressed as a transition between green and brown colour states (Kraus and Allison 2009). We can assume that in the long term, lighter individuals will be more prevalent in sunny habitats while darker individuals will be more prevalent in shady habitats. In the population, this leads to a favour for specific phenotypes that are more reflective of the environment and more protective from predators, due to natural selection. Long-term colour adaptation to local environments that evolved in response to natural selection is reported in lizards (*Phyrnocephalus versicolour*) (Tong *et al.* 2019), that exhibit geographically divergent body colourations to maximize camouflage against local backgrounds.

B. variegata is a long-lived anuran, and its maximum lifespan is between 5 and 23 years in the wild (Plytycz and Bigaj 1993; Hantzschmann *et al.* 2019; Di Cerbo *et al.* 2011), or 27 - 29 years in captivity, (in the absence of extrinsic mortality factors) (Mertens 1970; Abbühl and Durrer 1998), though there is still variability in life expectancy. Gene flow between neighbouring populations appears to be limited to less than 5 km (Hantzsmann *et al.* 2019). Hantzsmann *et al.* (2019) also showed that migration is limited to shorter distances in short-lived populations than in long-lived populations. Thus, life expectancy is another factor that can potentially play a role in the spread of the phenotype in a given environment, and it likely plays a role in the spread of favourable ventral spot patterns. Based on this hypothesis, we can assume that the structure and characteristics of a particular habitat play a role in the differences in spotting between populations that are more spatially separated. The population (i.e., the locality) has a significant influence on the distribution of properties of the individuals, indicating the substantial level of geographical differentiation among the populations (Radojičic *et al.* 2002).

The availability of food sources is also related to environmental conditions. Ogilvy *et al.* (2012) found that in some anurans, a diet fortified with carotenoids seems to have a strong impact on development and growth, but also on reproductive success and colouration of adult individuals. For instance, the colouration of the red ventral patch of *Bombina orientalis* is dependent on the supply of pigments in food and a lack of these carotenes under rearing conditions leads to a yellowish colouration (Steinicke 1976; Frost and Robinson 1984).

These results also suggest that differences such as food source variability, which vary between habitats, can lead to colour changes in frogs. Thus, the habitat may not only play an important role in the presence of the species in the locality (Csanády *et al.* 2020) but may also lead to a preference for other spot patterns that take into account environmental conditions.

Genetic diversity and population analysis

Studies dealing with genetic diversity and differentiation can not only offer basic information on ecology and evolution, but also provide background material for practical use in gene pool protection. The analysis of microsatellites and genetic diversity of *B. variegata* has not yet been processed. Current research on microsatellites (Stuckas and Tiedemann 2006; Hauswaldt *et al.* 2007) has focused on *B. bombina*. In our work we were able to amplify 5 and analyse 4 microsatellite loci, previously amplified in *B. bombina, B. variegata* and *B. orientalis* in the above mentioned studies. Stuckas and Tiedemann (2006) used samples from Denmark and Germany as study material and described 8 new microsatellite loci (7 of which were polymorphic) for the critically endangered *B. bombina* species. The observed heterozygosity produced values between 0.27 - 0.7 and did not differ significantly from the expected values (0.39 to 0.8), with the exception of locus Bob B13, (which we also used in our work), which yielded the largest deviation. The values for individual loci in *B.*

variegata, recorded by Stuckas and Tiedemann, are: Bob F22 - size 132 bp (in our results the locus was homologous with size 135 bp), Bob B13 - size 126 - 134 bp (our results 113 - 121 bp).

Hauswaldt *et al.* (2007), also analysed microsatellites. They described 9 new polymorphic microsatellite loci in *B. bombina*. Expected heterozygosity ranged from 0.47 to 0.91 and no significant deviation was reported. The results for locus Bob 1A in *B. variegata* are similar to ours; 5 alleles with a size of 320 - 354 bp were observed, which was comparative to the results of our study, where 7 alleles were present with a size of 322 - 353 bp. Finally, for locus Bob 5F Hauswaldt found 5 alleles measuring between 130 - 150 bp, compared to our observation of 9 alleles with a size of 115 - 147 bp.

The results from the analysis of microsatellites in *B. variegata* in Bukovské vrchy (Poloniny NP) are similar to these previous works. The observed heterozygosity (except for the Bob B13 locus) was not significantly different from expected heterozygosity, nor was there any significant deviation from the Hardy-Weinberg equilibrium. The trend in the Bob B13 locus towards lower observed heterozygosity could indicate a lack of homozygotes due to inbreeding, or selection against heterozygotes due to subpopulations (Wahlund effect). According to our averaged values, the observed heterozygosity of 0.5123 did not differ significantly from the expected heterozygosity of 0.5245, and thus the Wahlund effect did not appear to have a significant impact, as the HOBS value was not demonstrably lower than the HEXP value. Since the value of the inbreeding coefficient in the case of the Bob B13 locus was slightly higher than zero than in the case of other loci, we could therefore consider inbreeding as the more probable cause of the observed phenomenon. As the observed heterozygosity is high, we can assume that the originally isolated populations were mixed, which would correspond to the determined genetic diversity of *B. variegata* within the monitored population presented in this study. The allele number data (Table 5) show that for the Bob 5F and 1A loci, there are one or more particularly widespread alleles, respectively, and the remaining alleles are less common. This phenomenon could indicate that the early founders of the populations had a limited number of alleles (for example due to the bottleneck effect) and the newly formed alleles had not yet succeeded or could not expand sufficiently. Genetic diversity depends not only on the number of alleles, (which was sufficient based on our observations), but also on their frequency. Because alleles were not evenly distributed, we can assume that the genetic diversity of the population is smaller. As can be seen (Fig. 10), a phylogenetic tree constructed by the UPGMA method does not have the best explanatory value, as we worked with only four loci, of which the Bob F22 locus is monomorphic.

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