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A new method for quantitative pattern analysis applied to two European *Bombina* species

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This paper describes a new computer-aided digital image analysing method with self-developed modifications of public domain software (Scione Image 4.02b) for quantification of geometric and colour-defined characters of patches. We demonstrate the method on *Bombina bombina* and *B. variegata* and their natural hybrids, separating the two species and defining intermediate specimens according to their belly colour pattern. Belly images of 20 *B. bombina*, 20 *B. variegata* and five of their hybrids were processed and analysed, using four patch size- and shape-related characters (patch density, area ratio, ratio of mean patch area and mean patch perimeter, circularity) and three colour-related characters (red, green and blue components). Descriptive statistics (Student's *t*-test) and canonical variate analysis were used to discriminate among the three groups of specimens, and the results were compared with results based on independent genetic data. The two species significantly differed in all the geometric and two of the colour characters, and were clearly separated by the multivariate analysis, while the hybrid specimens had intermediate features.

Key words: Anura, *Bombina bombina*, *B. variegata*, Bombinatoridae, colour pattern mapping, hybrid zones, image analysis

INTRODUCTION

Pattern mapping is a commonly used method in herpetology (Donnelly et al., 1994), especially in capture–recapture studies, where analysis of patterns provides a cost-effective and non-invasive method for individual identification (Arntzen et al., 2004). Pattern analysis has been widely used for identification of individuals (Nilsson, 1954; Hagström, 1973; Sauer, 1994; Wright & Minott, 1999; Eitam & Blaustein, 2002; Bradfield, 2004), but characters derived from pattern can also be diagnostic for species identification and aid in detection, grouping and description of intra- or interspecific hybrid specimens and populations (Gollmann, 1984; Griffiths et al., 1987; Szymura & Barton, 1991; Brede et al., 2000). Distribution, position, connections and numbers of spots are quantifiable characters that are useful for correct pattern analysis. Computer-based image analysis is not only a technique applicable to large populations at low cost (Arntzen et al., 2004), but makes analysis of pattern fast, accurate and repeatable. Although digital image processing based on individual spot size measurement is a useful tool for morphological characterisation (Szalay, 2001; Szalay et al., 2001), its application in herpetology is still narrow (Streich et al., 1997; Hamilton, 2005).

Another challenging aspect of pattern analysis is the quantification of colour patches. Despite the limitations of colour analysis (Stevens & Cuthill, 2005), a variety of techniques has been used for the study of animal colours (Berggren & Merilä, 2004; Hamilton et al., 2005). Colour analysis requires standardized illumination at sampling, which is difficult to achieve under field conditions; however, examination of live specimens in the laboratory is invasive and costly.

Here we present a study that combines pattern mapping with computer-based image analysis, quantifying pattern and colour characteristics. We used an image analysing toolkit named "Scion Image" (Scion Co., Frederick, Maryland, USA), a popular freeware program often utilized in biological and biomedical investigations for quantitative description of morphological elements. We applied the method to populations of two European *Bombina* species (the fire-bellied toad *B. bombina* and the yellow bellied toad *B. variegata*), separating them according to the belly colour pattern and defining hybrid populations, and compared the results with those based on independent genetic data (Vörös et al., 2006). We also tested whether the calculated belly pattern size- and shape-related and colour characters are applicable for *Bombina* identification.

Bombina bombina, with a western and southern European distribution, and *B. variegata*, with an eastern and northern European distribution, form a narrow hybrid zone in central Europe (Arntzen, 1978). Isolated *B. variegata* populations can be found in low elevation mountains in Hungary and are surrounded by *B. bombina*, resulting in extensive interbreeding in the contact zones between the two species (Szymura, 1993; Vörös et al., 2006). The two species differ in morphology, electrophoretic markers, mitochondrial DNA and mating calls (Szymura, 1993). However, in contact zones they hybridize and produce fertile offspring, and the main diagnostic characters seem to be transitional.

While hybridization in *Bombina* has been thoroughly studied from genetic perspectives (Gollmann, 1984, 1987; Gollmann et al., 1988; Szymura & Barton, 1991; Nürnberger et al., 1995; Szymura, 1983, 1993, 1995; Kruuk et al., 1999; Szymura et al., 2000; Nürnberger et al., 2005;

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Spolsky et al., 2006), morphological approaches to characterization of hybrid individuals have proved difficult. The diagnostic characters distinguishing between the two species have been traditionally associated with the ventral colour pattern. This pattern usually consists of a predominantly black background with irregular patches varying in colour from bright red to light yellow. The differences between the two species lie in the ratio of the dark background to colour patch areas, in the colour of patches and in the relative position of colour patches (i.e. the pattern formed by the differential fusion of patches; Méhely, 1891).

Several classification systems have been developed for identifying and grouping *Bombina* individuals. It was Michalkowski (1958) who first studied the morphological characters of hybrid specimens, using the following characters: the dorsal papillae and the degree to which the belly was covered with colour patches as primary characters; isolation or fusion of the two main breast patches and the relation of patches on the boundary of the belly with the tights as secondary characters; and the arcuate black patches over the scapula and the white spots on the ventral side of the body as third-grade characters. The colour of ventral patches as a character in *Bombina* taxonomy was introduced by Stugren (1959), who used this character, together with the connection of patches and shape of dorsal papillae, to characterize pure and hybrid *Bombina* populations, and to group them according to given values varying from zero (pure *B. bombina*) to 12 (pure *B. variegata*). Stugren & Vancea (1968) recognized eight forms within *B. variegata*. These forms differ in the ratio of black and yellow pigmentation on the ventral surface, described as a percentage. Lác (1961) considered the warts on the skin as a primary character, and emphasized the colour and connection of ventral colour patches. According to the importance of certain characters, a formula was derived for each specimen. Three categories (typical forms, intermediate specimens and one species with characteristics of the other species) were specified. Szymura & Barton (1991) characterized individuals and populations with a hybrid index, based on 13 characteristics of the pattern of spots, depending on whether a particular spot was fused or not. Since *B. variegata* has many more colour patches on the belly than *B. bombina*, the average value of the characters was greater in *B. variegata*. The colour of the toes (dark or light) was also considered a diagnostic character. Gollmann (1984) introduced a classification system based on the presence or absence of the connections between 18 predefined colour patches. He produced an average value for each character to describe *Bombina* populations.

The aim of our study was to develop a new computer-based pattern analysis method, and to test it on the ventral colour pattern of the two European *Bombina* species. We analysed the diagnostic belly pattern of individuals from different populations belonging to the two species and their hybrids, and tried to identify the specimens using the belly-pattern-related characters. We used characters related to the size, shape, extension, number and colour of ventral patches located on the belly part of the ventral surface of toads. We calibrated the accuracy of the method with independent genetic data.

MATERIALS AND METHODS

Study material

In Hungary *Bombina variegata* lives in island-like mountainous habitats surrounded by *B. bombina*, which inhabits the lowlands. In the contact areas of their distributions, individuals of the two species interbreed and form hybrid zones. Hybridization between the two species has been reported from several areas of Hungary (Méhely 1904; Gollmann et al., 1988; Sipos, 1986; Gollmann, 1987). We obtained 20 specimens from pure *B. bombina* populations at three lowland localities (Dinnyés, Pilis, Őrség), 20 pure *B. variegata* specimens from four mountainous localities (Mecsek Mts, Őrség, Bátaapáti, Zemplén Mts), and five putative hybrid specimens from a hybrid area of the Bakony Mts (Table 1). The 40 pure specimens and one of the putative hybrids were previously studied and identified based on mtDNA data (Vörös et al., 2006), and the other four putative hybrids originated from the same hybrid area. *Bombina variegata* specimens were studied from two mitochondrial lineages (Alpine and Carpathian), both present in the Carpathian basin, and the hybrid specimen was identified based on the discordance of morphological and mtDNA data (Vörös et al., 2006). We used the mitochondrial results for *a priori* species identification, and the morphological data was compared to the genetic data in order to demonstrate the efficiency of our belly-pattern analysis method.

Table 1. Localities, number of specimens and GPS coordinates of localities of the three studied groups: *B. bombina*, *B. variegata* and the putative hybrids.

Species	Locality	*n*	Latitude	Longitude
Bombina bombina	Dinnyés: Dinnyési-fertő	8	47°11.202'	18°31.014'
	Őrség: Gyunác	6	47°00.078'	16°37.202'
	Pilis: Tó-lak	6	47°39.549'	18°58.211'
B. variegata	Bátaapáti	4	46°12.025'	18°36.422'
	Mecsek: Mt Jakab	8	46°05.354'	18°09.422'
	Őrség: Kercaszomor	6	46°46.477'	16°20.063'
	Zemplén: Sima-Baskó	2	48°12.221'	21°15.456'
B.bombina × *B.variegata*	Bakony Mts: Csehbánya	5	47°11.335'	17°41.012'

Ventral pattern photography and data collection

Specimens were laid on their back in a glass-covered box lined with a soft sponge. The toads were immobilized by gently pressing them with the sponge against the glass cover, and the belly was smoothed to prevent skin folding. The ventral pattern was recorded on a video-tape through the glass cover. Short shots (5–10 sec) were recorded from each individual by the first author with a commercial Sony 8 XR VHS C camera under ambient daylight. Labels identifying individuals and unit scales were recorded together with each specimen. The video-tapes were played and still images of each individual were selected at the most appropriate moment of the sequence, i.e. when the animal was immobile and the view of the belly was clear. Grayscale still images at 8 bit depth, 240×320 pixel size, were captured using a Fujitsu Siemens Pentium 4 computer with a Mercury TV tuner card. Digitized images were processed with Scion Image for Windows 4.02b (Scion Co., Frederick, Maryland, USA) image analysis software (a public domain tool available at www.scioncorp.com). In order to facilitate the processing of numerous samples, built-in Pascal-like macros were coded and implemented in the program by the authors (available upon request). The hardware requirements of the software are low. For the Macintosh platform we recommend NIH Image 1.5 or higher. This is the original clone of Scion Image and their macros are compatible with certain limitations.

Description of the method

The pattern sampling was divided into two parts: first we took the patch size measurements, then we measured the colour characters.

Patch size measurements. The first step was to select a region of interest (ROI) from the image of the ventral surface. Only this ROI was used for the following procedures, in a separate window. The exact outline of the abdomen was manually defined by a pentagon between the shoulders, the pelvic girdles and the cloacal region (Fig. 1). The segmentation of patches was identified on the basis of their grey level. A predefined range of grey levels appeared in the program as red, and it was manually adjusted with keystrokes until the optimal covering of patches was identified by the program. This is an important procedure, because improper selection of the range may cause fusion of patches or incorrect patch size measurements. For reduction of data noise, a default range (3–10,000 pixels) of spot sizes was given. This way, false patches due to glitter or optical contrasts could be eliminated from skin patches. To assist the user in defining spot segmentation, the previous views of the belly remained on the screen. The selected range of grayscale in the Look Up Table (LUT) (Fig.1) represented the spots as separately scattered areas. Five variables – total area of the belly, area of patches (in pixels), mean grey density level ("mean"), its standard deviation ("S.D.") and the perimeter of patches ("length" in pixels) – were measured for each individual by automatic calculation, and data were inserted into an ASCII text window ("Results") (Fig.1).

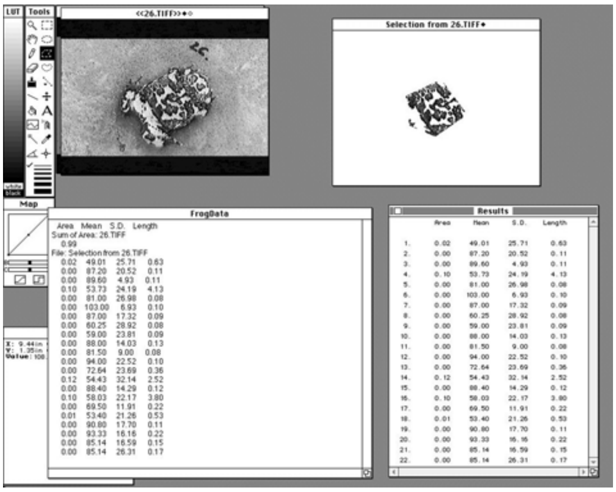

Fig. 1. Program window to illustrate the measuring process. Four variables are measured on the selected polygon (upper right) by an automatic calculation. These data are inserted into an ASCII text window (below left), then a Microsoft Excel compatible file (below right) is also created.

Numerical parameters can be separated from each other by any user-defined separator character; therefore a file structure compatible to Microsoft Excel can also be created ("FrogData") (Fig.1). It is important to note that area and perimeter were measured in number of pixels inside and around the spots, respectively.

With the help of three measured characters (total area of the belly, area of patches and perimeter of patches) we described the belly pattern of each specimen, and defined four additional, calculated characters to quantify overall belly pattern:

1) Patch density (PD): ratio of number of patches to the total area of the selected belly part.

2) Area ratio (AR): the ratio of the coloured parts to the black background on the belly.

3) Ratio of mean patch area and mean patch perimeter (RMPA): the smaller this value, the more irregular the patch is.

4) Circularity (CI): to what extent the shape of the patches corresponds to a circle, according to the formula below, where C = circularity, A = area of patches and P = perimeter of patches (C = 1 in the theoretical case of a circle.):

$C=(4\pi A)/P^2$

Every calculated character was measured in pixels. Since photos of toads were not taken at standard distance, for the comparison of samples we used only the four calculated, size-independent characters.

Colour characteristics. To describe colour characteristics the same software (Scion Image) was used. The image analysis was carried out separately from the patch size measurements, since this part of the analysis did not require macro-coded steps. Opening the photo file an indexed colour image and a grayscale three-layered R-G-B (red, green, blue, respectively) image appeared automatically. In the red layer of the grayscale image, optimal covering of spots was adjusted manually with associated

Table 2. Mean values, ranges and standard deviations for all variables in *B. bombina* and *B. variegata*. PD: patch density, AR: area ratio, RMPA: ratio of mean patch area and mean patch perimeter, CI: circularity, R: red, G: green, B: blue. See text for description of variables.

	B. bombina (*n*=20)				*B. variegata* (*n*=20)			
	Mean	Minimum	Maximum	SD	Mean	Minimum	Maximum	SD
PD	0.00772	0.0033	0.01756	0.00391	0.0016	0.0006	0.00326	0.00072
AR	0.30200	0.1816	0.44803	0.07299	0.5450	0.4016	0.69867	0.07282
RMPA	1.84687	0.8976	3.10705	0.58366	5.4111	2.9093	9.55901	1.69108
CI	0.00794	0.0035	0.01604	0.00324	0.0409	0.0128	0.09742	0.02246
R	18.66900	-18.5833	38.53333	16.36822	45.8950	28.7633	82.99333	11.39375
G	1.84417	-27.4300	26.60333	13.78535	36.1823	16.3733	77.04000	12.53670
B	-3.89950	-35.0733	53.74000	21.57596	-13.3368	-47.0567	30.23667	16.93074

keystrokes, and colour spots to be described were selected manually. We measured the R, G and B components separately on the three layers without changing the position of selected spots. Since recordings were taken under optically different circumstances, our image data varied in colours and brightness. For the estimation of different light parameters of illumination, the RGB value of the constant background (sponge) was used. Squares of the same size at three corners of the sponge were selected, and their RGB values were measured. By comparing the mean RGB values of the sponge and the belly patches, threshold was adjusted to maintain consistency.

Statistical analysis

We analysed variables (calculated characters) using descriptive statistics (mean, range, standard deviation) with the software Statistica 5.1 (StatSoft Inc., 1996). Comparing the variances of each variable an *F* test was performed in order to test the hypothesis that the two groups consisted of 20 *a priori* genetically identified specimens of *B. bombina* and 20 *B. variegata*. If the two variances were not significantly different, Student's *t*-test for independent samples was performed for each variable, in order to demonstrate the differences in belly pattern characters of the two species. If the variances of the two sets differed significantly, we applied a Welsch test instead of a *t*-test (Bányai & Barabás, 2002). Finally, canonical variate analysis (CVA) was carried out (SYNTAX 2000: Podani, 2001) on the whole dataset in order to discriminate between the three predefined groups: "pure" *B. bombina*, "pure" *B. variegata* and the putative hybrid specimens.

RESULTS

The mean values, ranges and standard deviations for all the variables are given in Table 2. PD was lower, AR was higher, RMPA was lower and CI was higher for *Bombina variegata*. R and G components were considerable higher and B lower for *B. variegata*.

The comparison of variables between the two "pure" populations using Student's *t*-test showed significant differences for all the four pattern size- and shape-related characters and for the R and G values at the 5% significance level. Only in B values did the samples not differ significantly (Table 3).

The canonical variate analysis showed a clear separation of the two "pure" groups along CV1, indicating remarkable differences in the majority of the characters (Fig. 2). Hybrid specimens from the Bakony area had an intermediate position between the two species, except for one specimen clustered within the *B. variegata* group. The first canonical axis explained 96.66% of the overall variation and the second axis explained the remaining 3.34%. Table 4 shows the correlations between the variables and each canonical variate axis. The first canonical variate (CV1) showed relatively uniform high loadings, and was negatively correlated with PD and positively correlated with AR, RMPA, CI and the R and G components

Table 3. Results of Student's *t*-test in case for every variable. PD: patch density, AR: area ratio, RMPA: ratio of mean patch area and mean patch perimeter, CI: circularity, R: red, G: green, B: blue. See text for description of variables.

Variable	*t* value	2-tailed *P* value
PD	6.851491	<0.001
AR	-10.5418	<0.001
RMPA	-8.90991	<0.001
CI	-6.15028	<0.001
R	-6.105	<0.001
G	-8.24	<0.001
B	1.538	0.132, NS

Table 4. *F*-ratio, coefficients for the canonical variables, and communalities for canonical variate analysis of *Bombina bombina* and *B. variegata*. 100% of the variance was displayed on two canonical axes.

Variable	*F* ratio	CV1	CV2	Communalities of variables for CV1 and CV2
PD	25.907	-0.790	-0.258	0.691
AR	46.529	0.879	0.351	0.896
RMPA	35.548	0.853	0.031	0.729
CI	20.744	0.751	-0.185	0.599
R	17.549	0.726	0.068	0.531
G	34.281	0.846	0.110	0.728
B	2.395	-0.254	0.509	0.323

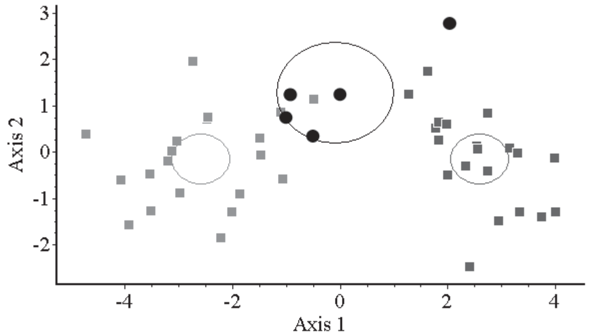

Fig. 2. Canonical variate analysis: plot of canonical scores for the three predefined groups: pure *B. bombina* (grey squares), pure *B. variegata* (black squares), and hybrid specimens (open circles). Confidence circles show the expected values of the statistical population with 95% probabilities.

of colour. On the second canonical axis (CV2), the greatest loading was for B and AR. *F*-ratio (ratio of variances between the groups) was high for all variables except B, demonstrating that the most discriminating characters give high correlations with the canonical variates.

DISCUSSION

Here we present a computer-based pattern-processing method for quantification and analysis of irregular colour patches. Our goal was to facilitate description of colour patches and to contribute to identification of species with complicated colour patterns. We applied our pattern description method to the belly pattern of the two European *Bombina* species, to demonstrate interspecific differences using characters derived from the belly pattern.

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 for analysing belly patterns of the two species, all based on similar characters: distribution, position, size and colour of the light patches. However, without exact quantification, these characters seem to be subjective. Our goal was to develop a method that can be used to quantify pattern and colour characteristics and make accurate measurements.

Our results showed that *B. variegata* has fewer, larger and more regular patches, and that the colour (RGB) composition of this species' pattern consists of higher red and green components, indicating a bright yellow colour. *B. bombina* has darker, orange spots, with lower values for red and green colour components.

The results also demonstrated that using our computerized method, coupled with multivariate statistical analysis, "pure" *B. bombina* and *B. variegata* populations can be distinguished on the basis of ventral colour pattern. All the characters derived from ventral colour pattern were useful for separation, except the least variable blue component of colour characteristics.

Ordination of individuals from the two pure and the hybrid populations along the two canonical axes showed that there was no overlap between the samples of the two species. The hybrid and putative hybrid specimens clustered in an intermediate position between the two species indicating intermediate characters. One specimen of the hybrid population fell into the *B. variegata* group; this seems to be a *variegata*-like hybrid. The other four specimens fell closer to the *B. bombina* group, representing *bombina*-like hybrids. The hybrid population chosen for the analysis (Csehbánya) is located at the border of the two species' range, in the low elevation Bakony Mts. The *Bombina* population in the pond of Csehbánya has been investigated by Sipos (1986) using morphometric characters, and both *bombina*-like and *variegata*-like hybrids together with the "pure" individuals of the parental species have been found in this mosaic hybrid area.

The analysis of ventral colour pattern with the help of our digital image processing method has enabled separation of two groups, one consisting of "pure" *Bombina bombina*, the other of "pure" *B. variegata* specimens, and placed specimens with intermediate characters between the two species. Although we demonstrated the efficiency of the method in two *Bombina* species, our image analysis can be applied to the assignment of other species, and for identification of individuals (e.g. in capture–recapture studies) with modifications of the macro.

With the presentation of this computer-based pattern analysing method we provide a tool to describe geometric and colour characters with several advantages. Applying this method, sampling is a simple, non-invasive and cost-effective procedure. The analysis can be easily and accurately repeated, and provides quantified data on irregular patches. It analyses not only the shape but also the colour scale of patches. As a future alternative, adaptation of similar macros is being tested with ImageJ 1.35r (Wayne Rasband, National Institutes of Health, USA), the platform-independent newer version of the Image software.

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