

Quantitative pattern analysis methodology in amphibians

Carlo M. BIANCARDI*, Anna Rita DI CERBO

Centro Studi Faunistica dei Vertebrati - Società Italiana di Scienze Naturali, C.so Venezia 55, 20121 Milano (MI)

* Corresponding author: cmbianca@alice.it

Riassunto. L'analisi quantitativa del pattern è utilizzata negli anfibi per il riconoscimento individuale e per la caratterizzazione di subpopolazioni e specie differenti. L'avvento dei metodi di analisi delle immagini digitali ha permesso il passaggio da una analisi basata su punteggi assegnati soggettivamente ad una estrazione ed elaborazione oggettiva delle informazioni contenute nei file digitali. Si rivede in maniera critica un metodo di analisi del pattern basato su rapporti di aree e perimetri delle parti di diverso colore, sulla loro forma e dimensione e si presenta inoltre un metodo di correlazione incrociata che tiene conto della posizione relativa delle diverse componenti del pattern. Il test sperimentale è stato effettuato su un campione di immagini di pattern ventrali di ululoni (genere: *Bombina*). Si propone la seguente procedura:

- i) Acquisire immagini con metodo standard, utilizzando una fotocamera su stativo con impostazioni fisse (e.g. risoluzione, sensibilità) o utilizzare una distanza fissa e un angolo di ripresa di 90°. Posizionare gli esemplari su terreno piano o in una scatola con un righello e un *checker* per il bilanciamento del colore.
- ii) Delimitare una Regione di Interesse seguendo punti di reperi anatomici. Scartare, o ridimensionare le foto distorte con *softwares* appositi (e.g. per studi di morfometria geometrica).
- iii) La *particle analysis* si basa sul calcolo di rapporti fra aree e perimetri. Vengono proposte e dettagliatamente descritte una serie di variabili standard: area ratio (AR), rapporto tra area media e perimetro medio delle particelle (RMPA), indici di circolarità (CI, HI) e indice di distorsione (EI).
- iv) Sebbene molte specie di anfibi non cambino in maniera significativa il *pattern* di colore nel corso della vita, in altre questo può accadere. È quindi meglio selezionare campioni con soggetti di età simile. Sugeriamo comunque di fotografare periodicamente gli animali nel caso di collezioni dedicate al riconoscimento individuale in studi a lungo termine.

Keywords. Pattern analysis, Individual identification, Amphibian colour pattern.

A colour pattern can be regarded as a mosaic of coloured spots or patches of various sizes, colours and shapes and their reciprocal position. Pattern mapping and analysis is a widespread method among herpetologist (e.g., Arntzen *et al.*, 2004; Donnelly *et al.*, 1994; Moon *et al.*, 2004; Davis *et al.*, 2007). It has been widely used for individual identification of different amphibian species (Winkler and Heunisch, 1997; Kopp-Harbenger, 1998; Eitam and Blaustein, 2002; Gamble *et al.*, 2008; Carafa and Biondi, 2004; Kenyon *et al.*, 2009), in many cases by manual comparison of photos with a reference photographic collection of individual colour patterns.

But colour pattern can also be a diagnostic character for populations, species and individuals (Gollmann, 1996; Szymura and Barton, 1991; Brede *et al.*, 2000; Carafa and Biondi, 2004; Voros *et al.*, 2007; Romano *et al.*, 2009; Angelini *et al.*, 2010). Quantitative analysis of the amphibian pattern is based on an array of values (scores). These scores can be subjectively assigned by the researcher, following a protocol or a template (Lac, 1961; Gollmann, 1984). This manual approach has been recently applied to discriminate between two species of *Bombina* in overlapping and hybridization zones (Ghiurca and Gherghel, 2007; Covaciu-Marcov *et al.*, 2009). However, digital images already contain a lot of information about position, size and colour of any single item. The image analysis is simply the extraction and elaboration of such information from pictures (Glasbey and Horgan, 1995). Aims of this work are to i) review the different methods of computer aided quantitative pattern analysis of amphibians described so far, ii) propose new method of analyses; iii) define standard methodologies.

Recent literature has been searched into the most popular scientific databases. The keyword used were “pattern analysis”, “colour pattern”, “amphibian colour”, “quantitative pattern”. The selected methodologies have been reviewed and applied, except for the colour analysis, to a sample of yellow bellied toads. Further, a new method proposed by the authors and applied to more extended samples is described (results in: Di Cerbo and Biancardi, this volume). The following software have been used for our analyses: Scion Image for Windows© (Scion Corporation, USA), with the self developed macros kindly provided by Dr. J. Voros (pers. comm); ImageJ (National Institute for Health, USA); LabView 2009© (National Instruments, USA).

There are three different approaches to the analysis of a set of digital images, choose among them often depends from their quality (Table 1).

Analysis	Image format	Color calibration	Rescaling	Topics	Advantages	Constraints
Particles	Binary	No	Not necessary	Take into account size and shape of the pattern elements, not the relative position.	Good images from different sources can be easily compared	Threshold: only well defined light/dark patterns can be analysed.

Particles & pattern	Binary, Greyscale or RGB	No	Yes	Compares the pattern elements according to their relative position.	Can be useful also for individual recognition	Pixel values assigned to 2-3 standard colour.
Colour characteristics	RGB	Yes	Not necessary	Same colour calibration for all images.	Variabilities due to differences in colour of the same patterns can be detected.	Colour Checker in all images should be included in the study design.

Tab. 1. Quantitative pattern analyses.

Variable	Description	Formula	Range	References
<i>A</i>	Particle area	$A = \iint f(x,y) dx dy$	-	Voros <i>et al.</i> , 2007
<i>P</i>	Particle perimeter	explained in the text	-	Voros <i>et al.</i> , 2007
<i>Mm</i>	Major and minor axes	explained in the text	-	Present <i>et al.</i> , 2007
<i>CI</i>	Circularity index	$CI = (4\pi A)/P^2$	0 to 1	Voros <i>et al.</i> , 2007
<i>HI</i>	Heywood index	Ratio between the actual perimeter of a particle and the theoretical perimeter of a circle axis of a particle	$> = 1$	Present work
<i>EI</i>	Elongation index	Ratio between major and minor axis of a particle	$> = 1$	Present work
<i>Npar</i>	Number of particles	Count	-	Voros <i>et al.</i> , 2007
<i>AR</i>	Area ratio	Ratio of the particle areas to the background	-	Voros <i>et al.</i> , 2007
<i>RMPA</i>	Ratio of mean patch area and mean patch perimeter. The smaller this value, the more irregular the particles are			Voros <i>et al.</i> , 2007

Tab. 2. Calculated variables in particle analysis.

1) Particles. This analysis, described by Voros *et al.* (2007) and applied on *Bombina bombina* and *B. variegata*, bases on the possibility to clearly distinguish light or dark

particles on a dark or light background. Pictures of bicolour patterns can be converted to binary images assigning the values 0 or 1 to the colour areas according to a threshold. This produces white particles on a black background. In figure 1, an example of a particle applied on our sample. The characteristic function of a particle in a binary image is:

- ⇒ $f(x,y) = 1$ for points that belong to the particle
- ⇒ $f(x,y) = 0$ for background points

The *area* (A) of a particle is given by its 0^{th} moment (Table 2), while its major and minor axes are given by an analysis of its 2^{nd} moment (I): $I = \iint r^2 f(x, y) dx dy$, where r is the distance between a point (x,y) and the axis we want to find. The *perimeter* (P) of a particle is obtained by a calculation which rough steps are: *i*) identify the pixel on the *edge* of a particle; *ii*) determine the contribution of each boundary pixel to the perimeter, which can be: $a = 1, b = \sqrt{2}, c = (1 + \sqrt{2})/2$; *iii*) sum all the contributions. The measures can be obtained in pixel or, using a conversion factor, in millimetres. To avoid the bias due to difference in size among sampled animals, the statistical analyses (ANOVA and Canonical Discriminant Analysis) have been performed on measure unit independent variables, like ratios and percentages (Table 2).

The Region of Interests (ROI), part of the image interested by the analysis, can be adapted to the characteristics of the pattern: circular, square, rectangular or pentagonal for belly pattern, circular for throat pattern, rectangular or irregular for dorsal or limbs pattern (e.g., Gollmann, 1996; Szymura and Barton, 1991; Brede *et al.*, 2000; Voros *et al.*, 2007; Romano *et al.*, 2009). For anurans belly pattern we suggest to select a ROI in two steps (Fig. 1):

- i) cut a rectangular image using the shoulders and the attachment of the hind limbs as reference points.
- ii) superimpose of a circular mask with diameter equal the major axis of the rectangle

Results will be statistically univariate or multivariate compared by individuals, populations, species (e.g., Voros *et al.*, 2007; Di Cerbo and Biancardi, this volume).

2) Pattern. As the subjects of the study could differ in size, to compare the relative position

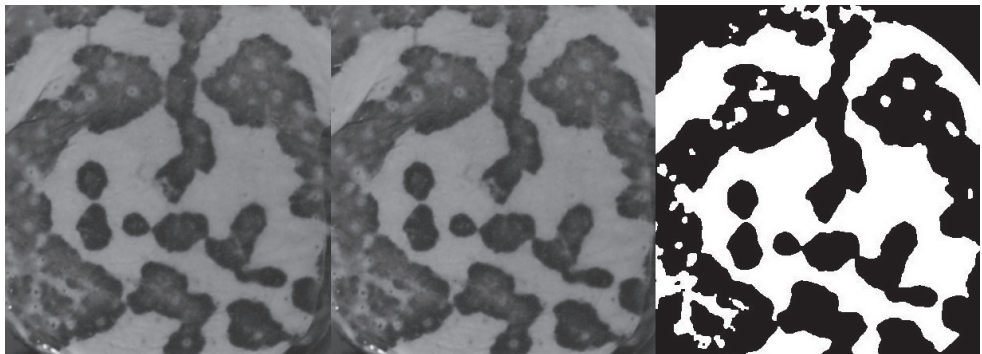


Fig. 1. Colour RGB, greyscale and binary image pattern. *Bombina variegata* ventral pattern.

of the elements of the pattern, images should be rescaled. To achieve this objective, we used a built-in function in LabView and tested the results: the number of particles and the area ratio did not change ($p = 1.000$). Recently, another method of reshape and rescale digital picture based on the morphometric geometry techniques has been described (Angelini *et al.*, 2010; Costa *et al.*, 2009). These authors recently presented an interesting analysis applied to spectacled salamanders (*Salamandrina perspicillata* and *S. terdigitata*), which considers each pixel position a variable and each pixel value an observation. Thus, multivariate analysis on the whole array can be performed. In order to have one bidimensional array the three-layer RGB images should be converted to single-layer greyscale. For interpopulation analysis, partial least squares method has been applied (Angelini *et al.*, 2010; Costa *et al.*, 2009).

The approach we suggest to compare two ROI's of the same size is a cross correlation. This is a standard method of estimating the degree to which two series are correlated. Consider two series $x(i)$ and $y(i)$ where $i=0,1,2...N-1$. The cross correlation r at delay d is defined as

$$r = \frac{\sum_i [(x(i) - mx) * (y(i - d) - my)]}{\sqrt{\sum_i (x(i) - mx)^2} \sqrt{\sum_i (y(i - d) - my)^2}}$$

Let x, y be the arrays of binary or greyscale values of two ROI's, r is the cross-correlation score of the patterns which can range from 0 (no correlation) to 1 (maximum correlation). When the delay d is set to 0, the images are totally superimposed, pixel by pixel. Delays ranging from -10 to +10 can be applied to both rows and columns of the arrays to correct for biases and errors in ROI's, selecting for the maximum cross correlation among the single analyses (Fig. 2). Correlation scores will be averaged and compared by populations with ANOVA.

Here, we didn't take in consideration the colour changes, and there is not room for a complete and critic analysis of this topic (e.g., the artefacts due to colour changes in preserved animals). However, in most cases the quantitative pattern analysis based on two or three fixed colour values can achieve the objective to characterize a population or species (Voros *et al.*, 2007; Romano *et al.*, 2009; Angelini *et al.*, 2010; Di Cerbo and Biancardi, this volume).



Fig. 2. Examples of binary patterns of *Bombina pachypus* (a, b) and the result of a superimposition of the two (c). Dark areas corresponds to the sum of black pixels in both images.

Being tested different methods, in conclusion we propose to follow these basic rules to achieve a standard methodology:

- 1) Standardize the image acquisition, using one digital camera mounted on a stand, fixed settings (e.g. pixels, light, sensitivity). In case of image taken in fields, use a standard distance and shot angle (90°). Put the specimen on a plain terrain or in a box, with ruler and colour checker.
- 2) Choose the ROI accordingly and cut the image following fixed mark points. Distorted images should be discarded or reshaped, Softwares designed for morphometric geometry can help (<http://life.bio.sunysb.edu/morph/>).
- 3) Particle analysis only require good digital images, even from different sources. Some commercial softwares, like the mentioned Scion and ImageJ, permit to get the basic numbers needed for the suggested analyses.
- 4) For rescaling and cross correlation or other advanced analyses, programming tools like LabView or MATLAB (Mathworks, USA) are needed.
- 5) About 48% of anurans have polymorphic color patterns (Kenyon *et al.*, 2010). Several species of Amphibians (e.g. *Bombina*, *Salmandrina*, *Salamandra*, *Litoria*) do not change significantly colour pattern during their life. However, others (e.g., *Triturus cristatus*, Arntzen and Teunis, 1993) could change it over time. In this case, it could be better select samples with similar size (age). Anyway, we suggest to photograph the single animal periodically when pattern analysis is applied to individual identification in longer-term studies.

Computer aided digital image processing can improve and increase the information of quantitative pattern analysis. However, great care should be given to the preparation and standardization of input images and the choose of the analyses approach.

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