# Quantitative analysis of the ventral colour pattern within the genus *Bombina*

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**Riassunto.** L'analisi quantitativa del pattern, secondo metodi messi a punto dagli autori sulla base della letteratura disponibile, è applicata a un campione di 268 immagini del pattern ventrale di ululoni del genere *Bombina*. Gli animali fotografati appartengono a *B. bombina* (Bb), *B. pachypus* (Bp), *B. v. variegata* (Bv), *B. v. scabra* (Bvs) e provengono prevalentemente dalle collezioni di quattro musei, un piccolo campione è stato fotografato sul campo. L'area ratio AR (intesa come rapporto fra l'area coperta dalle parti scure e quella della parte pigmentata in giallo-arancio) presenta differenze significative fra i quattro taxa, con una tendenza alla diminuzione proporzionale delle aree scure da Bb (AR = 70.3%) a Bv (AR = 49.3%), a Bp (AR = 44.7%) e a Bvs (AR = 22.7%). Nei confronti multipli queste differenze restano significative (ANOVA con Bonferroni: $p < 0.001$). Gli indici di circolarità (CI: $p < 0.001$), Heywood (HI: $p < 0.001$) e di distorsione (EI: $p < 0.05$) mostrano differenze significative fra *B. variegata* e *B. pachypus.* Le due specie si differenziano per la forma degli elementi scuri del pattern, più regolari e tendenti alla forma circolare in quella appenninica. Questi risultati, le analisi statistiche multivariate e la cross-correlazione dei pattern indicano una chiara distinzione di Bb dagli altri tre gruppi, una alta variabilità intraspecifica in Bv e un gradiente in diversi caratteri che segue un ordinamento Bv, Bp, Bvs, con quest'ultima maggiormente correlata con il congenere appenninico.

**Keywords.** Pattern analysis, *Bombina v. variegata*, *Bombina v. scabra*, *Bombina pachypus*, *Bombina bombina.*

Coloration and spatial colour pattern is one of the most frequently used characters for traditional animal identification at different taxonomic levels (e.g., Todd *et al.*, 2005) and for discrimination among populations (e.g., Costa *et al.*, 2008) and individuals (e.g., Carafa and Biondi, 2004). Toads of the genus *Bombina* present brightly coloured red-orange or yellow-and-black ventral patterns, which act as aposematic coloration. The patterns of the European species have been described: either as a predominantly black background with irregular patches varying in colour from bright red to light yellow (e.g., Voros *et al.*, 2007), or as a complex of grey or blackish spots and patches on a bright red or yellow background in *B. variegata* (e.g., Di Cerbo and Bressi, 2007); bluish-black spots on yellow-orange background in *B. pachypus* (e.g., Guarino *et al.*, 2007); red-orange-yellowish spots on black background in *B. bombina* (e.g., Sas *et al.*, 2005)

Quantitative characters associated to the ventral pattern, such as the ratio of the dark area to the bright coloured area, the actual colour of the patches, the quantity, shape and relative position of the patches, are considered diagnostic to distinguish the species *B. bombina* and *B. variegata* and their hybrids (Lac, 1961; Gollmann, 1984; Plaiasu *et al.* 2005; Sas *et al.*, 2005; Ghiurca and Gherghel, 2007; Voros *et al.*, 2007; Covaciu-Marcov *et al.*, 2009).

In Italy, lives the subspecies *B. v. variegata* and the endemic species Apennine yellow-bellied toad (*B. pachypus*), which is morphologically similar to *B. variegata*. The two species are vicariant, with the Po line acting as geographic barrier.

*Bombina pachypus* has been considered a sub-species of *B. variegata* for many years but, since last decades of the 20$^{th}$ century, it is considered a full species (Nascetti *et al.*, 1982; Fromhage *et al.*, 2004), Although its taxonomic position is still controversial and debated o (e.g., Hofman *et al.*, 2007; Zengh *et al.*, 2009).

Aim of our research is to compare the ventral pattern of the two yellow bellied toads which occur in Italy: *B.v. variegata* (following called with the acronym Bv) and *B. pachypus* (Bp), to verify and assess the presence of morphological differences and diagnostic characters between them.

Two small samples of European geographic distant from our Bv and Bp groups populations, *B. bombina* (Bb) and *B. v. scabra* (Bvs), have been analysed to have a more complete scenario. The former lives in Central and Eastern Europe and Balkans, the latter is a subspecies endemic of the Southern Balkans.

The study materials was a series of 268 digital images of the belly pattern in Tagged Image File Format (TIFF), obtained either by photos of museum specimens or by digitalization of a photographic archive of wild animals for individual recognition purpose (Table 1).

The preserved specimens were laid on their back in a glass-covered box lined with a syntetic soft sponge. The toads were immobilized against the glass cover taking care of smoothing the belly to prevent undesired skin folding (Voros *et al.*, 2007). The pattern shots were taken by a stand-mounted Nikon D80 with a 70 mm lens, images RGB 24 bit at 10M pixels were saved at a resolution of 300dpi. Archived photos were digitized by a scanner HP Scanjet G4010 as RGB 24 bit images at 300 dpi. The images were transformed in greyscale 8 bit by the extraction of the red plane and then in the final binary format by a manual-adjusted threshold.

The delimitation and shape of the Region of Interest (ROI) and the details of the quantitative pattern analysis methodology are described by Biancardi and Di Cerbo (this volume). In particular a calculated circular mask, centred on the belly, has been superimposed to each image in order to draw the contour of the ROI. The relative size and shape of yellow

| Species | Region | Source | Count |
|---|---|---|---|
| **Bombina pachypus** | Liguria (12) | Museum of Florence | 1 |
| | | Museum of Genoa | 6 |
| | | Museum of Turin | 5 |
| | Tuscany (18) | Museum of Milan | 5 |
| | | Museum of Florence | 13 |
| | Abruzzo (2) | Wild | 1 |
| | | Museum of Genoa | 1 |
| | Emilia Romagna | Museum of Florence | 2 |
| | Umbria | Museum of Genoa | 1 |
| | Campania (30) | Museum of Genoa | 5 |
| | | Museum of Turin | 25 |
| | Puglia | Museum of Florence | 2 |
| | Calabria (34) | Museum of Milan | 8 |
| | | Museum of Florence | 21 |
| | | Museum of Genoa | 5 |
| | | **Total *Bp*** | **101** |
| **Bombina variegata** | Lombardy (39) | Wild | 35 |
| | | Museum of Milan | 4 |
| | Trentino A.A. (23) | Wild | 4 |
| | | Museum of Milan | 3 |
| | | Museum of Florence | 3 |
| | | Museum of Genoa | 12 |
| | | Museum of Turin | 1 |
| | Veneto (44) | Museum of Milan | 1 |
| | | Museum of Florence | 3 |
| | | Museum of Turin | 40 |
| | Friuli V.G. | Museum of Florence | 25 |
| | | **Total *Bv*** | **131** |
| ***B.v. Scabra*** | Greece | Museum of Turin | 19 |
| | | **Total *Bvs*** | **19** |
| **B. Bombina** | Germany | Museum of Turin | 2 |
| | Moldova | Museum of Turin | 12 |
| | Romania | Museum of Turin | 3 |
| | | **Total *Bb*** | **17** |
| | Total Source | Wild | 40 |
| | | Museum of Milan | 21 |
| | | Museum of Florence | 70 |
| | | Museum of Genoa | 30 |
| | | Museum of Turin | 107 |
| | | **Total** | **268** |

Tab. 1. Sample size and distribution.

versus dark patches in the ventral pattern, considering dark particles on yellowish or orange background, have been investigated (Di Cerbo and Bressi, 2007). The measure unit used was pixel. To avoid influence of different scales in the photographic sample the calculated variables were either counts or measure ratios:

1. Number of particles (Npar)
2. Area Ratio (AR): the ratio of the particle areas to the background.
3. Ratio of mean patch area and mean patch perimeter (RMPA): the smaller this value, the more irregular the patch is.
4. Circularity index (CI): to what extent the shape of the patches corresponds to a circle, according to the formula $CI = (4\pi A)/P^2$, where A = area of patches and P = perimeter of patches. CI ranges from 0 to 1 (the latter value in the theoretical case of a circle).
5. Heywood index (HI): another circularity index often used in shape analysis. It represents the ratio between the actual perimeter of a particle and the theoretical perimeter of a circle of the same area. The greater the value of HI, the more irregular the shape is (HI = 1 in the case of a perfect circle).
6. Elongation index (EI): also called aspect ratio, is the ratio between length and width of a particle (EI = 1 in case of regular particles).

In order to avoid artefacts, particles cut by the contour of the ROI have been excluded from CI, HI and EI calculation. Npar and RMPA have been calculated also for yellow patches, while AR is complementary (AR dark + AR yellow = 1). Further, the ROI's were rescaled to a calculated standard dimension of 484x596 and a cross-correlation analysis has been performed to compare the relative position of the pattern elements (Biancardi & Di Cerbo, this volume).

Analyses were performed by means of the software LabView 2009 (National Instruments, USA) and the statistical package SPSS 18.0 (SPSS inc., USA).

Wild animals photo archive refers to *B. v. variegata* and *B. pachypus* individuals captured during previous researches (Di Cerbo, 2001).

An overview of the descriptive statistics is shown in table 2. All the variable average values are significantly different among the four populations (ANOVA: all $p<0.001$, except for EI $p<0.01$).

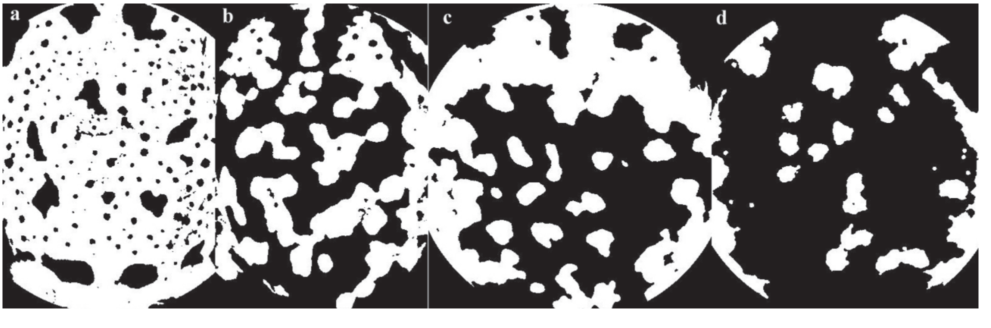


Fig. 1. Example of bellies ROI's. Black: yellowish background; White: dark particles. a) *B. bombina*; b) *B. v. variegata;* c) *B. pachypus*; d) *B. v. scabra.*

| | *B. variegata* (n=131) | | | | *B. pachypus* (n=101) | | | | *B. v. scabra* (n=19) | | | | *B. bombina* (n=17) | | | | ANOVA | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Background (yellow)** | **Mean** | **Min** | **Max** | **Std. Dev** | **Mean** | **Min** | **Max** | **Std. Dev** | **Mean** | **Min** | **Max** | **Std. Dev** | **Mean** | **Min** | **Max** | **Std. Dev** | **df** | **Partial eta squared** | **Power** | **Sig.** |
| **Npar** | 13 | 2 | 38 | 7 | 12 | 3 | 27 | 6 | 5 | 1 | 22 | 5 | 39 | 13 | 120 | 28 | 3 | 0.346 | 1.000 | *** |
| **RMPA** | 18.487 | 7.274 | 53.547 | 7.397 | 24.212 | 6.292 | 47.003 | 9.664 | 46.050 | 14.814 | 70.780 | 16.002 | 8.252 | 4.550 | 14.406 | 2.626 | 3 | 0.439 | 1.000 | *** |
| **Particles** | | | | | | | | | | | | | | | | | | | | |
| **Npar** | 12 | 2 | 57 | 7 | 17 | 3 | 43 | 7 | 30 | 12 | 63 | 15 | 5 | 1 | 14 | 4 | 3 | 0.338 | 1.000 | *** |
| **RMPA** | 14.432 | 5.824 | 45.790 | 5.077 | 14.365 | 5.556 | 26.601 | 4.382 | 7.719 | 4.744 | 14.524 | 2.494 | 44.001 | 12.898 | 74.561 | 16.931 | 3 | 0.604 | 1.000 | *** |
| **AR** | 0.493 | 0.290 | 0.635 | 0.077 | 0.447 | 0.240 | 0.643 | 0.095 | 0.227 | 0.108 | 0.536 | 0.109 | 0.703 | 0.538 | 0.826 | 0.082 | 3 | 0.521 | 1.000 | *** |
| **CI** | 0.643 | 0.160 | 0.966 | 0.128 | 0.728 | 0.444 | 0.929 | 0.082 | 0.727 | 0.554 | 0.868 | 0.093 | 0.652 | 0.274 | 0.973 | 0.234 | 3 | 0.108 | 0.999 | *** |
| **HI** | 1.337 | 1.017 | 2.678 | 0.215 | 1.224 | 1.038 | 1.535 | 0.099 | 1.223 | 1.109 | 1.378 | 0.089 | 1.340 | 1.016 | 1.912 | 0.300 | 3 | 0.091 | 0.995 | *** |
| **EI** | 2.530 | 1.584 | 5.216 | 0.635 | 2.320 | 1.755 | 4.304 | 0.310 | 2.323 | 1.940 | 2.788 | 0.241 | 2.112 | 1.595 | 3.356 | 0.450 | 3 | 0.062 | 0.949 | ** |

**Table 2. Descriptive statistics and ANOVA. (Significance: * = p<0.05; ** = p<0.01; *** = p<0.001)**

Tab. 2. Descriptive statistic and ANOVA. (Significance: *= p<0.05; **= p<0.01; **= p<0.001;).

Bonferroni correction have been applied to compare the taxa each other (Table 3). The number of particles and the area ratio between particles and background are significantly different in all the tests. However the three variables related to the particle shape (CI, HI and

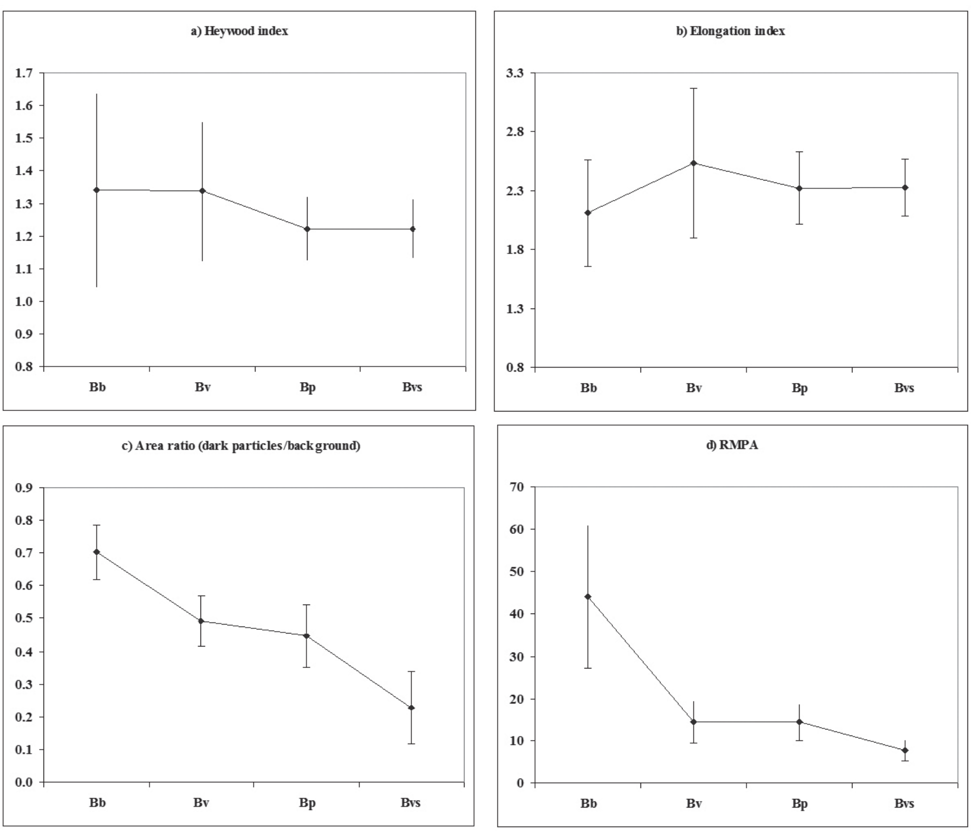


Fig. 2. Mean values ± st.dev. of four variables.

| Background (yellow) | Bv - Bp | Bv - Bb | Bv - Bvs | Bp - Bb | Bp - Bvs | Bb - Bvs |
|---|---|---|---|---|---|---|
| Npar | ns | *** | ** | *** | * | *** |
| RMPA | *** | *** | *** | *** | *** | *** |
| **Particles** | | | | | | |
| Npar | *** | ** | *** | *** | *** | *** |
| RMPA | ns | *** | *** | *** | *** | *** |
| AR | *** | *** | *** | *** | *** | *** |
| CI | *** | ns | * | ns | ns | ns |
| HI | *** | ns | ns | ns | ns | ns |
| EI | * | ** | ns | ns | ns | ns |
| **Pattern** | | | | | | |
| r mean | 0.25 | 0.16 | 0.26 | 0.17 | 0.29 | 0.19 |
| r max | 0.51 | 0.36 | 0.47 | 0.39 | 0.50 | 0.33 |

Tab. 3. ANOVA with Bonferroni correction and cross correlation coefficient *r*. (Significance as in table 2).

EI) are significantly different only between Bv and Bp, CI also between the two subspecies of *B. variegata* and EI between Bv and Bb. RMPA differences, which account for an average index of irregularity of the dark particles and the yellow/orange patches, are not significant only between the particles of Bv and Bp. A graphic representation of the mean values and their standard deviations is shown in fig. 2. Circularity refers to HI and RPMA to particles.

There is a clear trend of decrease of the area ratio from Bb to Bv, Bp and Bvs. The formers presenting a darker pattern than the latter's (Table 2).

The first canonical discriminant function, which explains the 79.4% of the total variance ($p < 0.001$), is highly correlated to AR, while the second function, which explains the 17.7% of the variance ($p < 0.001$), is correlated to the number of particles and EI. The third function explains the rest of the variance (2.9%; $p = 0.005$) and shows a correlation to the circularity indices. The discriminant functions were able to correctly classify 70% of original Bv and Bp cases, reaching 90% of correct predictions for Bb and Bvs. This overlap between Bv and Bp can be observed in fig. 3, where the first two canonical functions with the distribution and centroids of the four populations are plotted.

The cross-correlation coefficients (Table 3) are significantly higher among the groups Bp, Bv, Bvs, while Bb patterns appears to be weakly correlated to the others ($p < 0.001$). As

| Pattern | Bp | Bv | Bvs |
|---|---|---|---|
| r mean | 0.33 | 0.22 | 0.40 |
| r max | 0.67 | 0.59 | 0.61 |

Tab. 4. Intraspecific cross-correlation.

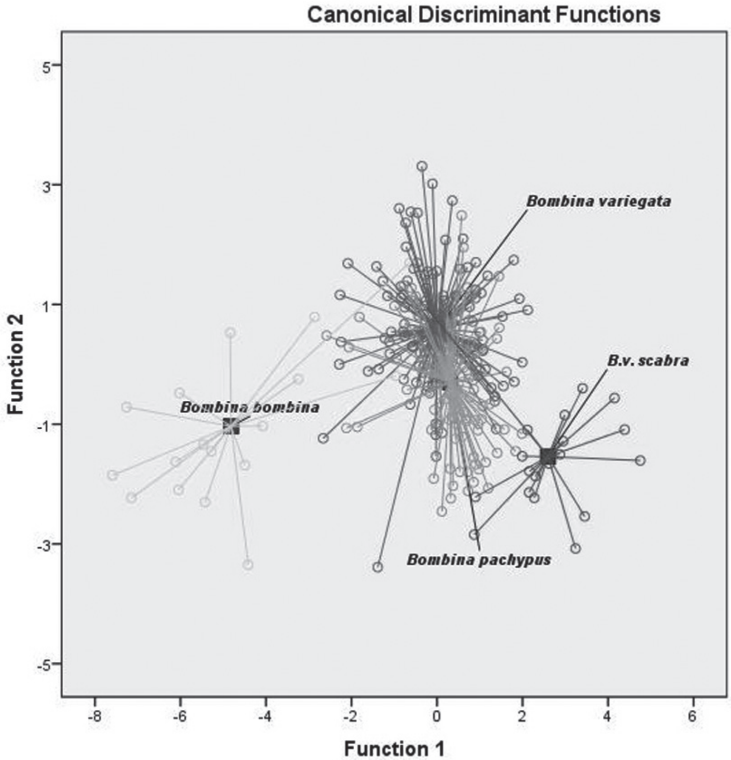


Fig. 3. Plot of the first two canonical discriminant functions with groups centroids.

expected, the intraspecific cross correlations are higher than the interspecific ones (Table 4).

However the mean cross correlation within Bv is smaller than between Bv and Bp or Bv and Bvs. This can be an indication of higher variability of the pattern among the geographic subpopulations of *B. variegata* with respect to the Balkan endemic subspecies and the Appennine species. Higher pattern correlation among Bp appears to be consistent with the scarce genetic variability among the Apennine populations, except for Calabria populations (Canestrelli *et al.*, 2006). However, further analyses will investigate intra- populations geographic variability of the pattern (Di Cerbo and Biancardi, in prep.).

The differences between the quantitative variables Npar, AR and RMPA of the patterns of *B. bombina* and *B. variegata* are confirmed even if, differently by Voros *et al.* (2007), we considered yellow as background. In the pattern of *B. bombina* the black pigmy prevails, while the yellow pigmy is predominant in *B. variegata* (Fuhn 1960). Sas *et al.* (2005) reported some differences between the two species regarding configuration of light spots (separated/united) on the ventral side, and Voros *et al.* (2007) reported significant differences in shape of them. The values of CI, HI and EI calculated on yellow spots in our sample (not reported in tables) gave significant differences as well (ANOVA, $p < 0.001$).

*B. pachypus* and *B. variegata* present significant differences in colour ratio, where the yellow areas are more extended in Bp, as well as in particle shape, being the particles in Bp more regular and circular-like than in Bv. However these two species appear to be more similar each other than to the other groups in this study. Further, the subspecies Bvs appears to be more similar to Bp than to the nominate subspecies for several characters, like the yellow extension, the shape of the particles and the cross correlation of the pattern. The ratio between black and yellow areas in *Bombina* shows a decrease gradient north to south, as observed in the eastern part of the distribution area of Bv (Stugren and Vancea, 1968).

The study of quantitative pattern differences can be a useful tool, used in synergy with genetics and phylogeography, to characterize populations and species. Deeper analysis extended to subpopulation will shed more lights in this matter (Di Cerbo and Biancardi, in prep.). Also an extension of the pattern analysis to throat and limbs is already planned.

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**References**

Biancardi, C.M., Di Cerbo, A.R. (2010): Quantitative pattern analysis methodology in amphibians. In: Atti VIII Congresso Nazionale SHI (Chieti, 22-26 settembre 2010), 383-390. Di Tizio, L., Di Cerbo, A.R., Di Francesco, N., Cameli, A., Eds, Iarieri Edizioni, Pescara.

Canestrelli, D., Cimmaruta, R., Costantini, V., Nascetti, G. (2006): Genetic diversity and phylogeography of the Apennine yellow-bellied toad *Bombina pachypus*, with implications for conservation. Mol. Ecol. 15: 3741-3754.

Carafa, M., Biondi, M. (2004): Application of a method for individual photographic identification during a study on *Salamandra salamandra gigliolii* in central Italy. Italian Journal of Zoology (Supplement) 2: 181-184.

Costa, C., Angelini, C., Scardi, M., Menesatti, P., Utzeri, C. (2008): Using image analysis on the ventral colour pattern in *Salamandrina perspicillata* (Amphibia: Salamandridae) to discriminate among populations. Biological Journal of the Linnean Society 96: 35-43.

Covaciu-Marcov, S.-D., Ferenti, S., Bogdan, H.-V., Groza M. I., Bata, Z. S. (2009): On the hybrid zone between *Bombina bombina* and *Bombina variegata* in Livada Forest, north-western Romania. Biharean Biologist 3: 5-12.

Di Cerbo, A.R. (2001): Eco-etologia di *Bombina v. variegata* (Linnaeus, 1758) in Lombardia

(Amphibia, Anura). Master thesis, University of Milan.
Di Cerbo, A.R., Biancardi, C.M. (in prep): Quantitative pattern analysis in *Bombina variegata* and *Bombina pachypus*: geographical and intra-population variability.
Di Cerbo, A.R., Bressi, N. (2007): *Bombina variegata*. In: Fauna d'Italia XLII. Amphibia, p. 280-287. Lanza, B., Andreone, F., Bologna, M.A., Corti, C., Razzetti, E., Eds., Calderini, Bologna.
Guarino, F.M., Picariello, O., Venchi, A., (2007): *Bombina pachypus*. In: Fauna d'Italia XLII. Amphibia, p. 276-280. Lanza, B., Andreone, F., Bologna, M.A., Corti, C., Razzetti, E., Eds, Calderini, Bologna.
Fromhage, L., Vences, M., Veith, M. (2004) : Testing alternative vicariance scenarios in Western Mediteranean discoglossid frogs. Molecular Phyl. Evol. 31(1): 308-322.
Ghiurca, D., Gherghel, I. (2007): Research upon the hybridization areas between *Bombina bombina* and *Bombina variegata* in the middle Siret River basin (Bacau and Neamt Counties, Romania). Herpetologica Romanica 1**:** 45-52.
Gollmann, G. (1984): Allozymic and morfological variation in the hybrid zone between *Bombina bombina* and *Bombina variegata*, (Anura Discoglossidae) in northeastern Austria. Z. Zool. Syst. Evol. Forsch. 22: 51-64.
Hofman, S., Spolsky C., Uzzel, T., Cogalniceanu, D., Babik, W., Szymura, J. (2007): Phylogeography of the fire-bellied toads *Bombina*:independent Pleistocene histories inferred from mitochondrial genomes. Mol. Ecol. (2007) 16: 2301-2316.
Lac, J. (1961): Rozsirenie kuncov (*Bombina bombina* L. a *Bombina variegata* L.) na slovensku a k problematike ich vzajomneho Krizenia. Biol. Pr. **7**(3): 5-32.
Nascetti, G., Vanni, S., Bullini, L., Lanza, B. (1982): Variabilità e divergenza genetica in popolazioni italiane del genere *Bombina* (Amphibia; Discoglossidae). Boll. Zool. 49 (suppl.): 134-135.
Sas, I., Covaciu-Marcov, S.D., Pop, M., Ile, R.-D., Szeibel N., Duma, C., (2005): About a closed hybrid population between *Bombina bombina* and *Bombina variegata* from Oradea (Bihor county, Romania). North-Western J. Zool. 1: 41-60.
Stugren B., Vancea S. (1968). Geographic Variation of the Yellow Bellied Toad (Bombina variegata) (L.) from the Carpathian Mountains of Romania and the USSR. Journal of Herpetology 2 (3-4): 97-105.
Todd, P.A., Ladle, R.J., Briers, R.A., Brunton, A. (2005): Quantifying two-dimensional dichromatic patterns using a photographic technique: case study on the shore crab (*Carcinus maenas* L.). Ecological Research 20: 497-501.
Voros, J., Szalay, F., Barabas, L. (2007): A new method for quantitative pattern analysis applied to two European Bombina species. Herpetol. J. 17: 97-103.
Zheng, Y., Fu, J., Shugiang, L. (2009): Toward understanding the distribution of Laurasian frogs: A test of Savage's biogeographical hypothesis using the genus *Bombina*. Mol. Phylogenet. Evol. 52: 70-83.