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Quantitative trait analysis of the *Bombina sp.* populations in the Transylvanian Hybrid Zone

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Abstract. In the present paper quantitative traits of *Bombina sp.* hybrids from a Transylvanian hybrid zone were studied. A number of 567 toads were studied for skeletal proportions (SVL, TFL), belly pattern (spot score) and other morphological characters. The distribution of the spot score index in the Vişea region shows a clear dominance of intermediate genotypes and leads to the conclusion that this hybrid zone is unimodal. Our data underlines that *Bombina variegata*-like hybrids have longer legs and are more adapted to puddle-like and terrestrial habitats. From the other morphological characters only the colour of the tip of toe was considered usable. The other characters like: the dorsal spots, the kidney shape spots and the existence of dorsal warts can be misleading, showing no correlation with spot score.

Key words: *Bombina* sp., hybrid zone, quantitative traits, spot score index.

Introduction

When two taxa hybridize, besides the parental taxa individuals with intermediate phenotype and genotype are observed in the hybrid zone. In earlier studies the scientists attempted to describe the intermediate phenotypes and to make deductions about the genetics of the hybridization. Since the discovery of the molecular methods, these quantitative traits got another meaning. The QTL

(quantitative trait loci) mapping is used to describe the genes underlying the differences between the pairs of species, through the estimation of the number and the chromosomal position of the traits. The importance of QTL from evolutionary point of view, is that they are particular traits affecting fitness.

The distribution range of the two fire bellied toads *Bombina bombina* and *B. variegata* overlap on the territory of Austria, Hungary, Ukraine, Poland, Croatia and Romania. *B. variegata* is patchily distributed in this region, with populations restricted to the mountainous areas. Wherever their distribution ranges adjoin in Central and Eastern Europe Areas, the two ecologically differentiated taxa hybridize and form fertile hybrids. Although the majority of individuals in the usually narrow contact zones (6-9 km wide) have hybrid genotypes, the two species do not fuse. This fact indicates, that a strong natural selection is maintaining the gene pools as separate entities. *B. bombina* and *B. variegata* differ in morphological, ecological and behavioural characters, most of which reflect their use of different breeding habitats (ponds versus puddles).

In the *Bombina* species quantitative traits such as: the egg size (Nürnberger et al. 1995, Köhler 2003), the developmental time to metamorphosis, the skin thickness (Nürnberger et al. 1995), the skeletal proportions, the belly coloration (Nürnberger et al. 1995, Vines 2002, Köhler 2003, Sands 2005) and the mating call (Sanderson et al. 1992, Nürnberger et al. 1995) were studied.

The clines in many traits were concordant with those in molecular markers. In the Krakow transect, the mating call components were concordant with the allozyme allele frequency clines (Sanderson et al. 1992). In the Pescenica transect the clines were also concordant for the quantitative traits (the belly pattern, the mating call cycle length, the skeletal proportions, the egg volume, and the skin thickness). Exception was observed in the development time to metamorphosis, where clines were displaced suggesting a strong, independent selection to the other traits (Nürnberger et al. 1995). Besides the concordant clines, strong linkage disequilibria between phenotypic traits in the centre of the zones was also found in the Polish and Croatian transects, probably generated by the mixing of immigrant adults in the centre of this cline (Sanderson et al. 1992, Nürnberger et al. 1995).

The distribution of quantitative traits in the Apahida hybrid zone (Sands 2005) appeared to be very similar to those seen across a cline between *Bombina bombina* and *B. variegata* in Croatia (Nürnberger et al. 1995, MacCallum et al. 1998), despite the different structures of the hybrid zones.

The aim of the present paper is to deal with the phenotypes of the studied populations, shedding light on the relationship among quantitative traits like: the spot score, the tibio-fibula length and others important in the study of *Bombina* hybrids. By examining and comparing the quantitative traits with the molecular markers, we can determine any differences in the selection pressures acting on these traits.

Material and methods

The study area

Our study area is located in the Apahida hybrid zone and lies around a small village called Vişea, at about 20 km north-east of Cluj (46°50'N, 23°47'E.), covering an area of 5x5 km² made up of either arable strip fields or pastures. Most of the aquatic habitats sampled for *Bombina* were on agricultural land, in the vicinity of the village or in the woods.

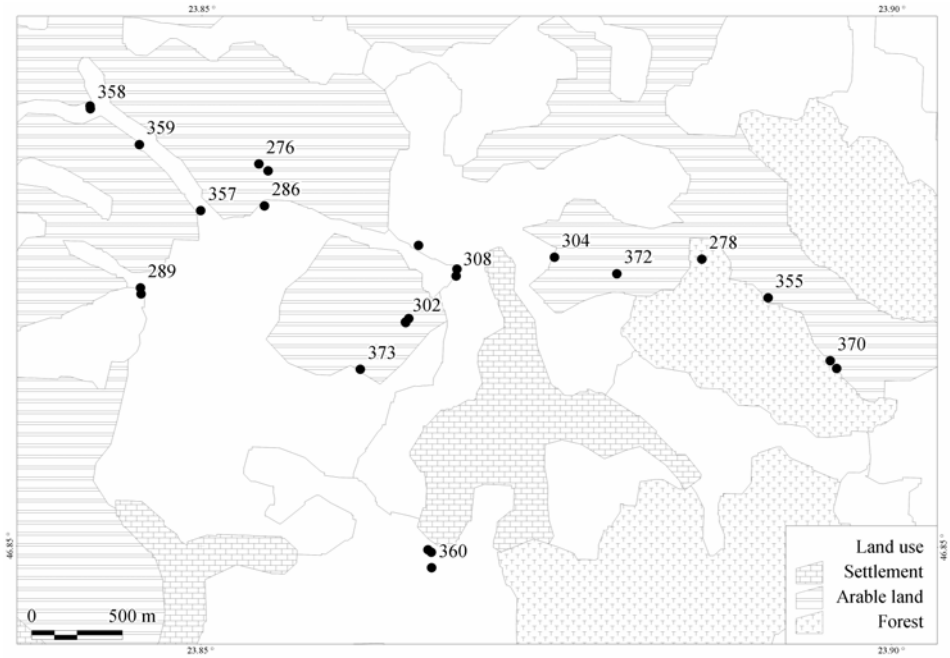

Figure 1. Map of the sampled aquatic sites and the land use around Vişea.

Data collection on animals

The results presented in this study were collected over two field seasons: 2001-2002, between mid April and mid June. The toads were collected from aquatic habitats (Fig. 1.). The animals were processed on the field. Toads were caught by net or by hand from the water surface. They were anaesthetised in 2% MS222 (3-aminobenzoic acid ethyl ester). The effect of MS222 lasted for 10-15 minutes, during this time the belly pattern of the individuals was scored, measured and photographed.

Spot score

The underside coloration of *Bombina* varies between the species. Szymura and Barton (1991) recognized that these morphological characters are determined genetically and they can be used to approximate an individual's genotype state. In order to obtain the so called spot score, one needs to record whether the yellow to red spots are connected to each other (1) or not (0) over ten critical points along the ventral side of a toad. The critical points are shown on the Figure 2. Thus, the spot score varies from 0 (pure *B. bombina*) to 10 (pure *B. variegata*).

Other morphological and morphometric characters

The presence (1) or absence (0) of quantitative traits such as: the dorsal spots, the dorsal warts, the kidney-shaped spots and the nuptial pads were registered, as well as the colour of the tip of toe. These morphological characters were used earlier in the morphological studies made on *Bombina* by Michalowski (1958, 1961), Stugren (1959) and Madej (1964). The snout-vent length and the tibio-fibula length were also measured, because it is known that the leg length varies between the two *Bombina* species (Nürnberger et al. 1995).

Statistical analysis

Correlation and regression analysis were used to determine the evidence of a relationship between two variables. To make basic statistical analyses (regression analysis, correlation analysis) and graphics, programs like SPSS 11.5 and Minitab were used.

The multinomial (polytomous) logistic regression model was used for hybrids as basic outcomes. Five morphological characteristics were considered (x_i): one continuous (SVL), three binary discrete variables (Warts, DS, TT) and one trinomial variable (KSH). We assume that the probability of a toad belonging to a certain group, given the predictor variables as a linear combination of $x_i\beta_m$, where the vector

$\beta_m = (\beta_{0m} ... \beta_{im} ... \beta_{Im})$ includes the intercept β_{0m} and is the coefficient for the effect of x_i on outcome m. The coefficients for the effect of the different predictor variables on the probability of belonging to the *B. variegata* and *B. bombina* were separately estimated as follows:

$$\Pr(y_i = m \mid x_i) = \frac{\exp(x_i \beta_m)}{\sum_{j=1}^{J} \exp(x_i \beta_j)},$$

where *m* represents the two purse species. The slope of regression lines was tested against the null hypothesis of $\beta = 0$ with the standard Z-test (Long 1997).

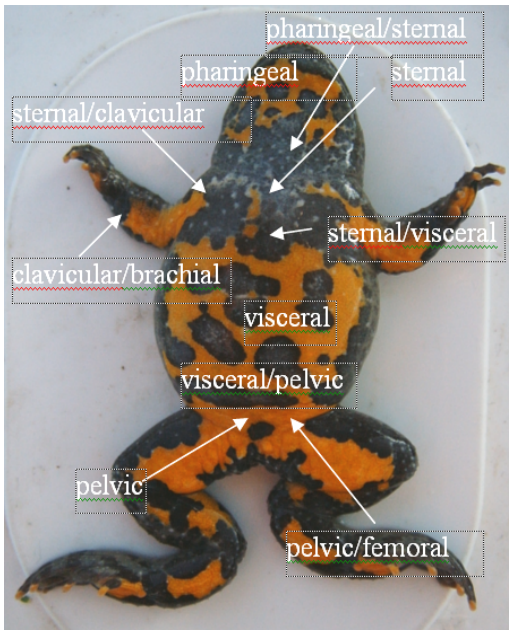

Figure 2. Spot score index (1-pharingeal, 2-pharingeal/sternal, 3-sternal, 4-sternal/clavicular, 5-clavicular/brachial, 6-sternal/visceral, 7-visceral, 8-visceral/pelvic, 9-pelvic, 10-pelvic/femoral)

The assessment of the model performance was made by the area under the Receiver Operating Characteristic curve (AUC). AUC is a good model for model accuracy and it is recommended for two class classifiers (Fielding and Bell 1997, Jesús and Ángel 2004). AUC varies between 1 and 0.5, where 1 denotes a perfect fit of the model compared to the observed incidence and 0.5 denotes no fit and bad models.

The Goodness-of-Fit of the model was also tested by the Pearson statistic as follows:

$$X^2 = \sum_{i=1}^{N} r_i,$$

where r_i represents the Pearson residual for the i^{th} observation. Model comparison was made using the Bayesian information criterion (BIC), the preferred model having the lowest value of the criterion. The difference in the BICs value for the two models indicates which model is more likely to be used for the data. Absolute differences in BIC values ($|\Delta_{BIC}|$) between 0-2 suggest weak, 2-6 positive, 6-10 strong and >10 very strong evidence of favouring one model to the other.

Results and discussions

SVL distribution and TF length of analysed toads

The majority of individuals captured and analysed during the field seasons were adults (513). Only a small number (54) of juveniles was observed (Figure 3.). The maximal body length in our study area was 50 mm, found in two individuals (a male in 2001 and a female in 2002). In Poland, the biggest yellow bellied toads had a body length of 51-55 mm (Plytycz and Bigaj 1993).

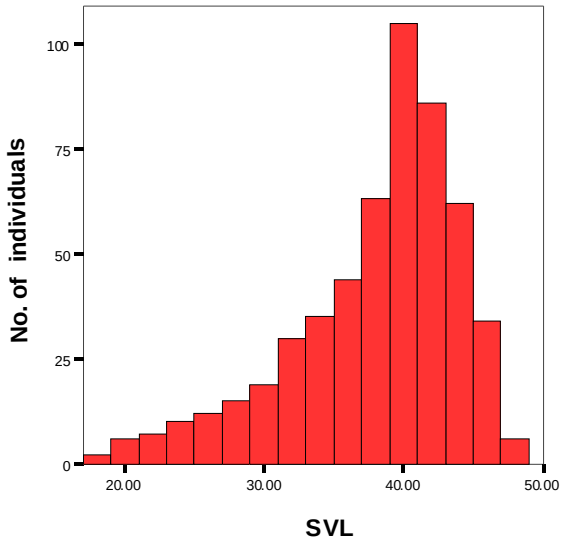

Figure 3. The snout-vent length distribution of the studied toads

Regarding the snout-vent length of the captured individuals, in the females the body length was shorter (38.9 cm, CI 95%: 38.2-39.6 cm) than in the males (40.0 cm, CI 95%: 39.6-40.6 cm). A difference of approx. 1-1.5 mm was found in a hybrid zone from Ukraine near Uzhgorod, studied by Piálek and Novotná (1992), where the snout-vent length of the studied males was 41.65 mm (SD=4.44, n=33), while the SVL of the females was 40.26 mm (SD=3.6 mm, n=41).

The yellow-bellied toads have longer legs due to the habitat preference (Fuhn 1960, Michalowski 1961, Nürnberger et al. 1995). In the study of Nürnberger et al. (1995) on quantitative traits in a *Bombina* hybrid zone from Croatia, 12 skeletal measurements were taken, from which the two dominant ones were tibiofibula (r=0.61) and femur (r=0.56).

We observed a significant negative correlation (r=-0.107, at 0.05 level, n=566) between the spot score and the snout-vent length/tibio-fibula ratio (SVL/TF) (Figure 4). This means that the TF of the yellow bellied toads and the *B. variegata*-like hybrids is longer, the taxon being more adapted to puddle-like habitats than the fire-bellied toads and the *B. bombina*-like hybrids.

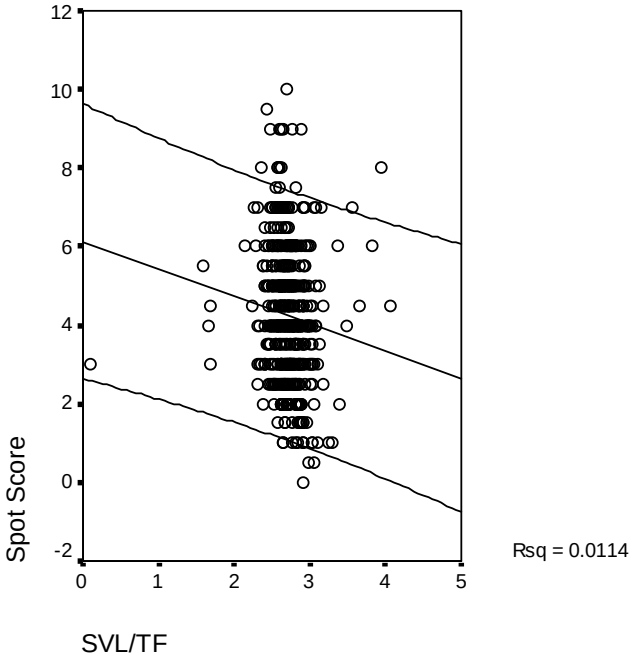

Figure 4. Regression of spot score versus SVL/TF

Distribution of the spot score index

The belly pattern can be used to approximate an individual's genotype state. A total number of 542 individuals from 29 habitats were scored. The distribution of the spot score index shows that in our study area there were more individuals showing an intermediate spot score (Figure 5.). The juvenile spot score shows a slight shift to the *B. variegata*-like end of the index compared with the adult spot score (mean 4.2 in adults vs. 4.6 in juveniles).

Even in the lack of genetic data, we can use the spot score data to make conclusions about the hybrid zone around Vişea, because the spot score index is highly concordant with the molecular markers (Szymura and Barton 1991, Nürnberger et al. 1995).We tested statistically whether there is a difference in the spot score index between adults and juveniles. The juvenile spot score values differ significantly (T-test, p=0.048) from the adult aggregation spot score. Therefore we suppose that the hybrid zone is changing in time. The spot score of adults is lower, because there are more *bombina* alleles in the adult aggregation than in the juvenile. In order to understand this result, we suppose that an introgression of *bombina* alleles occurs, from the big lake on the top of the hill to the valley. An extrinsic selection pressure acts and *variegata* alleles are selected due to the local constraints, causing the lack of *bombina* alleles in juvenile aggregations.

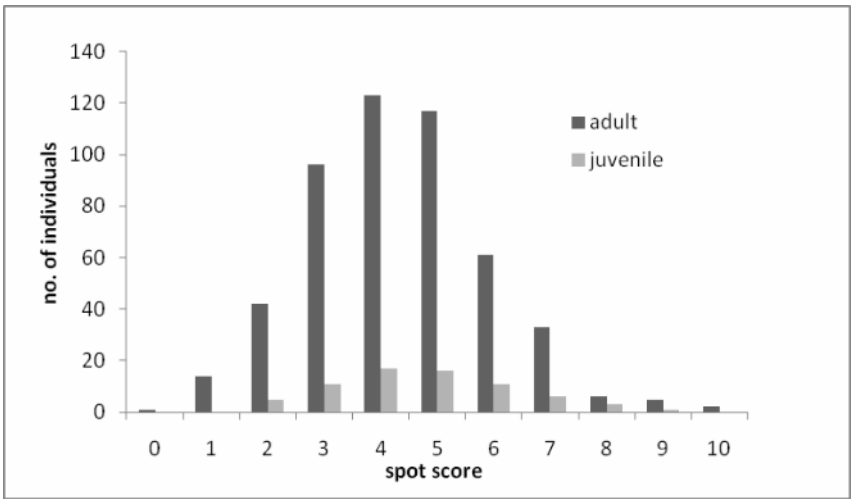

Figure 5. The distribution of the spot score index for adult and juvenile individuals (on the Y coordinate the number of individuals and on the X coordinate the mean spot score are represented)

Other morphological characters

The values of the morphological characters such as: the dorsal spots, the dorsal warts, the tip of toe and the kidney-shaped spots were summarized and then correlated with the spot score index of the toads. The correlation coefficient between the morphological traits and the spot score index is r = 0.227, being significant at the 0.01 level (Figure 6.).

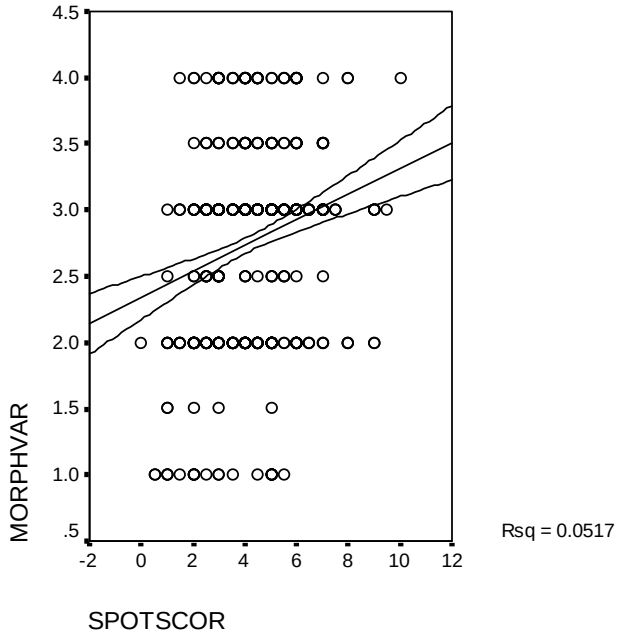

Figure 6. Regression of the morphological variables and the spot score.

In order to find out which morphological character is more important, the mean data per site for the morphological characters were correlated with the mean spot score per site. Only one character, the colour of the tip of toe is correlated with the mean spot score (r=0.602, p=0.01, n=22). There is no significant correlation between the spot score and the other three characters: the dorsal warts, the dorsal spots and the kidney shaped spots.

The Goodness-of-Fit Pearson test indicates that there is insufficient evidence for the model, not fitting the data adequately (χ^2 = 800.757; DF= 802; P=0.506).

Pairwise comparisons suggest that SVL is longer in *B. bombina* than in hybrid toads and slightly shorter in *B. variegata,* both differences being significant from a statistical point of view.

One categorical predictor, the tip of toe (TT), was scored statistically significant. The incidence of TT is significantly smaller in *B. bombina,* while in *B. variegata* the incidence of TT is more frequent, but the difference is not significant (Table 1).

To assess the model suitability we have also compared the two parental species. The model has a fair performance (AUC=0.795). We recorded again a significant difference regarding the body length and the tip of toe. The model showed extremely low sensitivity, while the specificity is satisfactory. The removal of the non significant terms from the model led to a BIC value of 232.002 for the full model and to 221.9 for the simplified model (Δ_{BIC}=10.102).

Table 1. Pairwise comparison of the studied characters
(SVL – snout-vent length, Warty – existence of dorsal warts, DS – dorsal spots,
TT – tip of toe, KS – kidney-shaped dorsal spots)

	SpScore		**Coef.**	**SE**	**z**	**P>|z|**	**95% Conf.Interval**	
B. bombina								
	SVL		.0497564	.0183335	2.71	0.007	.0138233	.0856895
	Warty		-.0735901	.2580269	-0.29	0.775	-.5793135	.4321333
	DS		-.1684063	.5369715	-0.31	0.754	-1.220851	.8840385
	TT		-1.616586	.2270214	-7.12	0.000	-2.061539	-1.171632
	KS		.4127701	.264778	1.56	0.119	-.1061853	.9317255
B. variegata								
	SVL		-.0476697	.0229793	-2.07	0.038	-.0927083	-.002631
	Warty		.5765801	.4279816	1.35	0.178	-.2622483	1.415409
	DS		-.5930754	.6200893	-0.96	0.339	-1.808428	.6222774
	TT		.9725166	.619539	1.57	0.116	-.2417575	2.186791
	KS		-.1365339	.4260483	-0.32	0.749	-.9715732	.6985055

The pairwise comparison of the studied characters strengthens our results, that the colour of the tip of toe is the most important morphological character. The snout-vent length of the individuals was proved to be significant, the *Bombina*

bombina individuals being larger than the hybrids and the *Bombina variegata* individuals.

The use of the colour of the tip of toe as a morphological character in distinguishing the *Bombina* species was proposed by Stugren (1959) and later improved by (Gollmann 1984 cited in 1987a, 1987b). The latter found that the yellow coloured first finger in hybrids shows a black ring.

In spite of the broad use of morphological classification in hybridization studies, we consider of low importance the characters like: the dorsal spots, the kidney shape spots and the existence of dorsal warts.

Conclusions

The age structure of the *Bombina* populations in the Vişea region shows a lack of juveniles. This is strongly influenced by the exogenous factors such as: the habitat quality and the weather conditions (e.g. the drought in the two years of study).

A negative significant correlation exists betweeen the SVL/TF ratio and the spot score, which means that the *Bombina variegata*-like hybrids have longer legs and are more adapted to puddle-like and terrestrial habitats.

The distribution of the spot score index in the Vişea region shows a clear dominance of intermediate genotypes and leads to the conclusion that this hybrid zone is unimodal.

From the other morphological characters only the colour of the tip of toe was considered usable. The other characters like: the dorsal spots, the kidney shape spots and the existence of dorsal warts can be misleading, showing no correlation with spot score. Therefore we do not recommend their use for hybridization studies.

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