FLUCTUATING ASYMMETRY AS A TOOL IN IDENTIFYING POPULATION STRESS IN HUNGARIAN POPULATIONS OF *BOMBINA BOMBINA*, *B. VARIEGATA* AND THEIR HYBRIDS

PLĂIAŞU, R.[1,3], VÖRÖS, J.[2] and BĂNCILĂ, R.[1,3]

[1]*"Emil Racoviţă" Institute of Speleology of the Romanian Academy*
13 Septembrie Road, No. 13, 050711, Bucharest, Romania, E-mail: rodica_plaiasu@yahoo.com
[2]*Department of Zoology, Hungarian Natural History Museum*
H-1088 Budapest, Baross u. 13, Hungary, E-mail: jvoros@nhmus.hu
[3]*University Ovidius Constanţa, Faculty of Natural Sciences*
Aleea Universităţii, No. 1, 900470, Constanţa, Romania

Hybridization can occur under natural conditions among well-differentiated species and may affect the developmental stability of hybrids. In the present study, we investigated the effect of interspecific hybridization between *Bombina bombina* and *B. variegata* on fluctuating asymmetry (FA) of forelimb, femur, tibia and foot. The hybrids did not show higher levels of FA compared to the parental populations for either investigated traits. This suggested that the effect of hybridization on FA in the analyzed traits is negligible or overwhelmed by other factors. A significantly increased FA was found in the *B. bombina* populations when compared to *B. variegata*, which can be attributed to low pressure of natural selection in these populations.

Key words: interspecific hybridization; *Bombina*; fluctuating asymmetry

INTRODUCTION

Interspecific hybridization can occur under natural conditions among well-differentiated species. The study of hybrids can offer valuable information on adaptation and speciation since they are also exposed to selection pressure (SCHWENK *et al.* 2008). Hybrids genotypes are sometimes fitter than the parental genotypes, but most models of hybridization considered, on average, hybrids less fit than the parents (BARTON 2001).

Genetic incompatibilities in the hybrids caused by the coexistence of genomes from two different species may destabilize their ability to develop an optimal phenotype due to increased developmental instability (DI) (ANDERSEN *et al.* 2006, CARREIRA *et al.* 2008). DI is the inability of an organism to buffer its development against environmental and genetic disturbances. One of the commonly used tools to estimate developmental instability in animals includes fluctuating asymmetry (FA), defined as random non-directional deviations from bilateral symmetry of the body (VAN VALEN 1962).

The European fire-bellied toads, *Bombina bombina* (LINNAEUS 1761) and *Bombina variegata* (LINNAEUS 1758), occur in Eastern and Northern Europe and

Western and Southern Europe, respectively. Their present distribution is the result of recent postglacial colonization. The geographic ranges of the two species meet along several thousand kilometers in Central Europe. Although these species have adapted to different breeding habitats they hybridize in the contact zone (SZYMURA 1993).

In this study, using both recent and historical population samples from the Herpetological Collection of the Hungarian Natural History Museum, we test the usefulness of FA in detecting shifts in developmental stability by focusing on populations from a naturally occurring hybrid zone between *B. bombina* and *B. variegata* in Hungary. We hypothesize that hybrids should present increased developmental instability because of disruption of genomic coadaptation.

MATERIALS AND METHODS

The material used in this study consisted of 232 preserved adults specimens of *B. bombina* (n = 108), *B. variegata* (n = 106) and of their hybrids (n = 18) from the Herpetological Collection of the Hungarian Natural History Museum (Table 1). The hybrids were identified according to morphological characters and previous knowledge on the genetics of the species (VÖRÖS *et al.* 2006).

Four measurements: forelimb, femur, tibia and foot were taken on the left and right side of each specimen. Measurements were taken exclusively by one of the authors (RP) to avoid possible inter-observer variability (LEE 1990).

Data analysis

Each measurement was repeated for each trait four times on both left and right side in 148 individuals and two times for the rest of the individuals. On the basis of the four repeated measurements a data set of 1184 measurements for each metric trait (two sides × four repeated measurements × 148

Table 1. The number of individuals and sex of *Bombina* sp. individuals from Herpetological Collection of the Hungarian Natural History Museum.

Taxa	Locality	Sex	
		M	F
B. bombina	Balaton	10	12
	Mátra Mts	27	13
	Budapest	24	15
	Tokaj	5	2
B. variegata	Bakony Mts	7	5
	Zemplén Mts	61	10
	Mátra Mts	5	3
	Mecsek Mts	11	4
Hybrids	Jósvafő	13	5

individuals) was obtained. For each trait a two-way mixed model ANOVA with individual as random factor and side as fixed factors were carried out. FA is measured by the side * individual interaction, directional asymmetry (DA) is expressed by the main effect for sides and measurement error (ME) is expressed by the residual term (PALMER & STROBECK 1986, PALMER 1994). Antisymmetry (AA) was examined using Kolmogorov–Smirnov tests of frequency distributions of right-left differences compared to an expected normal distribution (PALMER & STROBECK 1986). When AA was detected, data were Box-Cox transformed (AITKEN *et al*. 1989). The association between FA and size was tested using the linear regression of the signed asymmetry (R–L) on character size defined as ([Ri+Li]/2), for each metric trait.

For each trait FA was calculated based on the average value of the two replicate measurements following the formula:

$$|R–L|/[(R+L)/2]$$

where *R* and *L* are the measurements on the right and the left body side. The denominator corrects for trait size in order not to weight traits with respect to their absolute length.

The influence of sex and population on metric FA was tested with a nonparametric Kruskal–Wallis test. As the mean asymmetry did not differ between populations or between gender data was pooled over populations and gender for each pure species and hybrids, respectively. We also used the nonparametric Kruskal-Wallis test to compare the FA measures in the two parental toad species and their hybrids. When significant differences between the three groups were found paired comparisons were done using a Mann–Whitney U test. Statistical analyses were carried out using SPSS software package (SPSS Inc., Chicago, IL, USA).

RESULTS

Significant FA that exceeded the measurement error was detected for all metric traits whereas no significant DA was found for either trait (Table 2).

The frequency distributions of right-left differences deviated significantly from normality and the unsigned FA was significantly correlated with trait size (Table 3).

Significant differences were found between parental species and hybrids for asymmetry of femur ($\chi^2 = 17.33$, df = 2, P < 0.01) and tibia length ($\chi^2 = 8.786$, df = 2, P < 0.05) whereas forelimb ($\chi^2 = 2.923$, df = 2, P = 0.232) and foot length ($\chi^2 = 0.733$, df = 2, P = 0.693) asymmetry showed no significant differences between the three groups.

Mann–Whitney U test for paired differences indicated that femur asymmetry differed significantly between hybrids and *B. bombina* (n =18 hybrids, n = 108 *B. bombina*, Z = –2.740, P < 0.01) with a higher asymmetry in *B. bombina* (Fig. 1a) and between the two parental species (n = 106 *B. variegata*, n = 108 *B. bombina*, Z = – 3.656, P < 0.01), femur asymmetry being higher in *B. bombina*. Multiple comparisons between the three groups for tibia asymmetry showed a significant differ-

Table 2. Two-way mixed model ANOVA for testing the significance of between-side * individual interaction (FA), between-side variation (DA) and between-replicates variation (ME) for the four measured traits. The statistical significance was computed after Bonferroni correction.

Trait	Fluctuating asymmetry				Directional asymmetry				Between replicate variation	
	df	MS	F	P	df	MS	F	P	df	MS
Forelimb	147	0.72	5.918	< 0.01	1	0.333	0.463	0.497	868	0.122
Femur	147	0.837	5.443	< 0.01	1	1.351	1.615	0.206	878	0.154
Tibia	147	1.284	6.349	< 0.01	1	0.199	0.155	0.694	878	0.202
Foot	147	2.23	11.05	< 0.01	1	10.618	4.761	0.124	868	0.202

Table 3. Means of the left-right differences ± standard error (SE), with Kolmogorov-Smirnov test and mean trait size values and correlation coefficients of left-right differences with mean trait size. The statistical significance was computed after Bonferroni correction.

Trait	(R-L)			[(R+L)/2]		
	Mean±SE	K-S	P	Mean±SE	r	P
Forelimb	0.416±0.420	6.446	< 0.01	13.921±0.249	0.833	< 0.01
Femur	13.921±0.249	2.418	< 0.01	14.160±0.171	0.287	< 0.01
Tibia	–0.037±0.071	3.669	< 0.01	13.581±0.147	0.310	< 0.01
Foot	0.050±0.157	3.908	< 0.01	22.334±0.211	0.432	< 0.01

ence only between the pure species (n =106 *B. variegata*, n = 108 *B. bombina*, Z = –2.962, P < 0.05), with a higher asymmetry in *B. bombina* (Fig. 1*b*). FA in metric trait did not significantly differ among populations or gender in none of the parental species or in their hybrids (Table 4).
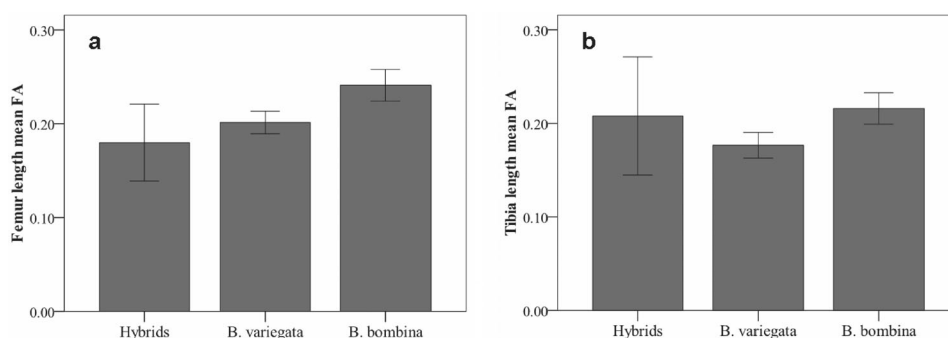
Fig. 1. Mean asymmetry compared among the two pure species and their hybrids for: a) femur and b) tibia length. Bars represent 95% confidence intervals

Table 4. Kruskal–Wallis non-parametric tests for differences in metric FA among populations and gender for the two parental species (*B. variegata* and *B. bombina*) and their hybrids.

			χ^2	df	P
B. bombina	Population	Forelimb	6.193	3	0.103
		Femur	1.389	3	0.708
		Tibia	6.415	3	0.093
		Foot	2.041	3	0.564
	Gender	Forelimb	0.15	1	0.698
		Femur	0.325	1	0.568
		Tibia	0.018	1	0.895
		Foot	1.879	1	0.17
B. variegata	Population	Forelimb	7.803	3	0.253
		Femur	4.335	3	0.631
		Tibia	11.05	3	0.087
		Foot	7.915	3	0.244
	Gender	Forelimb	0.278	1	0.598
		Femur	1.152	1	0.283
		Tibia	0.755	1	0.385
		Foot	0.884	1	0.347
Hybrids	Gender	Forelimb	0.197	1	0.657
		Femur	1.076	1	0.3
		Tibia	0.002	1	0.961
		Foot	0.411	1	0.521

DISCUSSION

Our hypothesis that hybridization implies higher FA values was not confirmed. The studied populations (except hybrids group) are situated in area with different condition and level of human impact nevertheless no significant differences in FA existed between populations of the same species. This suggests that observed FA is not attributable to different local condition.

Endogenous factors as level of genetic divergence among species and exogenous factors as age and stability of hybrid zone could influence variation in hybrids FA (LAMB *et al*. 1990). Considering old time of genetic divergences between parental species (HOFMAN *et al*. 2007) and recent origin of the main hybrid site (GOLLMANN *et al*. 1988, LÁC 1961) the hybrids should have lower developmental stability. A possible explanation of the results involves endogenous selection against hybrids evidenced through high embryonic and larval mortality (KOTEJA 1984).

Thus, the adult hybrids are the genotypes minimally affected by hybridization. Studies on hybrids offspring viability had contrast results attributed to different lineage of *B. variegata* occurring in the hybrid areas (NÜRNBERGER *et al*. 1995). The *B. variegata* populations from the Aggtelek hybrid site belong to a very divergent mitochondrial lineage (Carpathian line) (VÖRÖS *et al*. 2006). However, selection pressures would induce marked reduction in the occurrence of incompatible combinations of alleles in the first generation and reduced frequency of these in the following hybrid generation (NÜRNBERGER *et al*. 1995).

A surprising result concern high levels of FA in *B. bombina* populations. The two species have different ecological requirement. *B. bombina* reproduces in semipermanent ponds whereas *B. variegata* is a specialist for ephemeral breeding sites. The last species is better adapted to dispersal because realize permanent movements through the landscape to locate reproduction sites and has robust skeleton (CZOPKOWA & CZOPEK 1955, YANCHUKOV *et al*. 2006, HARTEL 2008). *B. variegata* tadpoles also grow and develop faster than *B. bombina* tadpoles. Therefore *B. variegata* individuals are under higher natural selection pressure, this possible explain why their levels of FA were lower than in *B. bombina*.

There is evidence for other anuran species that the elements important in locomotion mechanism have lower level of FA than other traits (SÖDERMAN *et al*. 2007).

Also, this is the proximate explanation for the significantly femur asymmetry between hybrids and *B. bombina*. In the hybrid areas there is an adult habitat preference for mating site: *B. variegata*-like hybrids were frequently found in puddles and *B. bombina*-like hybrids are more often found in ponds (MACCALLUM *et al*. 1998, VINES *et al*. 2003). Thus, adults could be forced to move over greater distances to find the preferred habitat.

In conclusion, the results of this study do not support the idea that FA is higher in hybrids than in parental populations. This suggested that the effect of hybridization on FA in the analyzed traits is negligible or overwhelmed by other factors.

*

REFERENCES

ANDERSEN, D. H., PERTOLDI, C., LOESCHCKE, V. & SCALI, V. (2006) Developmental instability, hybridization and heterozygosity in stick insects of the genus Bacillus (Insecta; Phasmatodea) with different modes of reproduction. *Biological Journal of the Linnean Society* **87**: 249–259.

AITKEN, M., ANDERSEN, D., FRANCIS, B. & HINDE, J. (1989) *Statistical modeling in GLIM*. Oxford, University Press Oxford, 374 pp.

BARTON, N. H. (2001) The role of hybridization in evolution. *Molecular Ecology* **10**: 551–568.

CARREIRA, V. P., SOTO, I. M., FANARA, J. J. & HASSON, E. (2008) A study of wing morphology and fluctuating asymmetry in interspecific hybrids between Drosophila buzzatii and D. koepferae. *Genetica* **133**: 1–11.

CZOPKOWA, G. & CZOPEK, J. (1955) The vascularization of the respiratory surfaces in Bombina variegata. *Bulletin of the Polish Academy of Sciences* **3**: 313–318.

GOLLMANN, G., ROTH, P. & HÖDL, W. (1988) Hybridisation between the fire-bellied toads Bombina bombina and Bombina variegata in the karst regions of Slovakia and Hungary. *Journal of Evolutionary Biology* **1**: 3–14.

HARTEL, T. (2008) Movement activity in a Bombina variegata population from a deciduous forested landscape. *North-Western Journal of Zoology* **4**: 79–90.

HOFMAN, S., SPOLSKY, C., UZZELL, T., COGĂLNICEANU, D., BABIK, W. & SZYMURA, J. (2007) Phylogeography of the fire-bellied toads, Bombina: independent Pleistocene histories inferred from mitochondrial genomes. *Molecular Ecology* **16**: 2301–2316.

KOTEJA, E. (1984) *Embryonic mortality of the fire-brllied toad (Bombina bombina), the yellow-bellied toad (Bombina variegata), and their hybrids*. Jagiellonian University, Cracow.

LÁC, J. (1961) Rozsirenie kuncov (Bombina bombina L. a Bombina variegata L.) na slovensku a k problematike ich vzajomneho Krizenia. *Biologické Práce* **7**: 5–32.

LAMB, T., NOVAK, J. M. & MAHONEY, D. L. (1990) Morphological asymmetry and interspecific hybridization: a case study using hylid frogs. *Journal of Evolutionary Biology* **3**: 295–309.

LEE, J. C. (1990) Sources of extraneous variation in the study of meristic characters: the effect of size and of inter-observer variability. *Systematic Zoology* **39**: 31–39.

MACCALLUM, C. J., NÜRNBERGER, B., BARTON, N. H. & SZYMURA, J. M. (1998) Habitat preference in a Bombina hybrid zone in Croatia. *Evolution* **52**: 227–239.

NÜRNBERGER, B., BARTON, N., MACCALLUM, C., GILCHRIST, J. & APPLEBY, M. (1995) Natural selection on quantitative traits in the Bombina hybrid zone. *Evolution* **49**(6): 1224–1238.

PALMER, A. R. & STROBECK, C. (1986) Fluctuating asymmetry: measurement, analysis, patterns. *Annual Review of Ecological Systematics* **17**: 391–421.

PALMER, A. R. (1994) Fluctuating asymmetry analysis: A primer. Pp. 335–364. *In:* MARKOW, T. A. (ed.): *Developmental Instability: Its Origins and Evolutionary Implications*. Kluwer Academic Publishers, Dordrecht.

SCHWENK, K., BREDE, N. & STREIT, B. (2008) Hybridization in animals – Extent, processes and evolutionary impact. *Philosophical Transactions of the Royal Society B* **363**: 2805–2961.

SÖDERMAN, F., VAN DONGEN, S., PAKKASMAA, S. & MERILÄ, J. (2007) Environomental stress increases skeletal fluctuating asymmetry in the moor frog Rana arvalis. *Oecologia* **151**: 593–604.

SZYMURA, J. M. (1993) Analysis of hybrid zones with Bombina. Pp. 261–289. *In:* HARRISON, R. (ed.): *Hybrid zones and the evolutionary process*. Oxford University Press, New York.

VAN VALEN, L. (1962) A study of fluctuating asymmetry. *Evolution* **16**: 125–142.

VINES, T. H., KÖHLER, S. C., THIEL, M., GHIRA, I., SANDS, T. R., MACCALLUM, C. J., BARTON, N. H. & NÜRNBERGER, B. (2003) The maintenance of reproductive isolation in a mosaic hybrid zone between the fire-bellied toads Bombina bombina and B. variegata. *Evolution* **53**: 1876–1888.

VÖRÖS, J., ALCOBENDAS, M., MARTÍNEZ-SOLANO, I. & GARCÍA-PARÍS, M. (2006) Evolution of Bombina bombina and Bombina variegata (Anura: Discoglossidae) in the Carpathian Basin: a history of repeated mt-DNA introgression across species. *Molecular Phylogenetics and Evolution* **38**: 705–718.

YANCHUKOV, A., HOFMAN, S., SZYMURA, J. M., MEZHZHERIN, S. V., MOROZOV-LEONOV, S. Y., BARTON, N. H. & NÜRNBERGER, B. (2006) Hybridization of Bombina bombina and B. variegata (Anura, Discoglossidae) at a sharp ecotone in western Ukraine: Comparisons across transects over time. *Evolution* **60:** 583–600.