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There is no heterotic effect upon developmental stability in the ventral side of the skull within the house mouse hybrid zone

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Abstract

Fluctuating asymmetry (FA) of a body is expected to be related to an organism's developmental instability. We studied patterns of FA in the ventral side of the skull along a transect across the central-European portion of the hybrid zone between two house mouse subspecies, *Mus musculus musculus* and *Mus musculus domesticus*. We found that FA was not significantly different between introgression classes relative to differences between localities within those classes and that the within-class differences were much larger than differences between individual-signed asymmetries within localities. However, if year was added to the same analysis as another factor, FA was not significantly different among localities within the same introgression class. When individual asymmetries were plotted against individual hybrid indices, hybrids appeared more asymmetric than individuals from outside of the zone. Thus contrary to previous studies, we did not find lower FA indicating heterotic effect in hybrids for the traits studied. It is suggested that the impact of hybridization on FA in the ventral side of the mouse skull is negligible or overwhelmed by other factors.

Introduction

Hybrid zones between related taxa are in the focus of evolutionary biologists as they offer an excellent occasion to study genetic barriers between natural populations; in particular, how they arise, what their nature is and what consequences they may have. One of intensively studied evolutionary phenomena is the impact of hybridization on developmental instability (DI), i.e. the sensitivity of development to intrinsic random perturbations (see Van Dongen, 2006 and references therein for discussion of the definition).

At first sight, it seems impossible to measure DI as we cannot run the same ontogeny multiple times, but fortunately there are some ways to circumvent this problem. The usual one is comparison of identical structures on both sides of a bilaterally symmetric body (Van Valen, 1962). They can be considered independent results of the same ontogeny as they are formed on the basis of the same genetic information and in the same environment, and thus only random differences between them should appear. The left–right differences that are symmetrically distributed around zero mean are referred to as *fluctuating asymmetry* (FA) whereas *directional asymmetry* (DA) is defined by nonzero mean of symmetrical distribution, i.e. one side being consistently larger than the other. Under specific conditions (defined, for instance, in Klingenberg, 2003), FA corresponds exactly to the random variation and can be therefore used as a measure of DI whereas DA is considered undesirable in this context as it indicates that some nonrandom differences are present. Unfortunately, there is no consensus how to treat traits exhibiting DA (cf. Palmer, 1994; Klingenberg & McIntyre, 1998; Klingenberg, 2003). Here we use average asymmetry as a measure for the nonrandom part, deviations normally distributed around this being considered as FA (see also Leamy, 1984; Palmer & Strobeck, 1986), although this relies on the assumption

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of no inherent variation for the particular left–right deviations. Actually, this assumption may be violated even for a trait exhibiting perfect FA provided the inherent differences are normally distributed in a population around zero mean (Palmer & Strobeck, 1992).

A third type of left–right variation is antisymmetry, which is characterized by bimodality of the signed (L–R) distribution. In this case, presence of nonrandom variation is supposed as well, but its statistical correction is much more difficult or virtually impossible and such traits were suggested to be omitted in the studies of DI (Palmer, 1994).

FA has been the focus of numerous studies trying to evaluate its relation to the intensity of stress or to individual fitness in a wide range of taxa. However, the results of these studies are rather equivocal (Leung & Forbes, 1996; Tomkins & Simmons, 2003; Pertoldi *et al.*, 2006a; Van Dongen, 2006). There are examples of significant correlations of FA with the level of environmental stress (Lens *et al.*, 1999; Leung *et al.*, 2000; Oleksyk *et al.*, 2004), genetic stress (Leamy *et al.*, 2001; Radwan, 2003; Pertoldi *et al.*, 2006b) or with various components of individual fitness (Martín & López, 2000; Polak *et al.*, 2002; Fréchette *et al.*, 2003). Other studies, however, failed to find such correlations (Bourguet, 2000; Vøllestad & Hindar, 2001; Breuker & Brakefield, 2002; Martin & Hosken, 2002; Kruuk *et al.*, 2003; Stige *et al.*, 2006) or questioned their evolutionary significance (Santos, 2001; Polak & Starmer, 2005). The genetic basis of FA has also been intensively investigated but generally only low heritabilities were found (Fuller & Houle, 2003; Leamy & Klingenberg, 2005). There is, however, increasing evidence that genetic basis of FA may be epistatic rather than additive (Leamy *et al.*, 2000, 2002, 2005) which is also in agreement with theoretical predictions (Klingenberg & Nijhout, 1999; Klingenberg, 2004).

The hybrid zone between two subspecies (or species, according to some authors, e.g. Sage *et al.*, 1993) of house mice, *Mus musculus musculus* and *Mus musculus domesticus*, in Europe yields an excellent opportunity to assess a potential impact of hybridization on DI. The zone runs across the Jutland peninsula and from East Holstein in Germany through central Europe and the Balkans to the Black Sea coast (Boursot *et al.*, 1993; Macholán *et al.*, 2003, 2007). Both direct and indirect evidence suggests post-zygotic reproductive isolation between the two taxa (Payseur *et al.*, 2004; Britton-Davidian *et al.*, 2005; Raufaste *et al.*, 2005; Macholán *et al.*, 2007), especially in the sex chromosomes (Vanlerberghe *et al.*, 1988; Tucker *et al.*, 1992; Dod *et al.*, 1993; Payseur *et al.*, 2004; Macholán *et al.*, 2007). We can expect three possible effects of hybridization on the level of FA in the mouse hybrid zone: either hybrids may benefit from increased heterozygosity leading to lower FA or suffer from disruption of coadapted gene complexes resulting in higher FA (Alibert & Auffray, 2003); finally, hybridization may have no measurable impact on FA.

Alibert *et al.* (1994, 1997) assessed FA of six teeth parameters both in wild-captured and laboratory-bred *musculus/domesticus* hybrids and concluded that FA (and hence DI) is decreased in hybrid animals, interpreting this as a consequence of increased heterozygosity. In laboratory-bred hybrids, Debat *et al.* (2000) found the same for FA studied on two-dimensional (2D) projections of the dorsal side of the skull.

Here, we adopted a procedure based on powerful tools of geometric morphometrics described in Klingenberg & McIntyre (1998) and Klingenberg *et al.* (2002) and analysed variation in FA along the central-European transect across the *M. m. musculus/M. m. domesticus* hybrid zone. For measuring FA, we chose the ventral rather then dorsal side of the mouse skull as a higher number of reliably scored landmarks can be found on this side. Moreover, digitizing landmarks in three dimensions decreased potential ambiguities in their position.

It is shown in this study that, contrary to the results of previous studies, there is no evidence for a heterotic effect of hybridization upon developmental stability along the central-European transect across the hybrid zone, the level of FA being slightly higher in hybrids than in genetically pure individuals.

Materials and methods

Mice

In total, skulls of 288 house mice (137 males and 151 females) were measured. The mice were trapped along a 110-km long by 40-km wide transect across the *M. m. musculus/M. m. domesticus* hybrid zone, running from eastern Bavaria (Germany) to western Bohemia (Czech Republic) (Fig. 1). In a few cases, mice from geographically close sites were pooled in order to increase the sample size. Thus 22 groups were created, referred to as 'localities' throughout the paper (Table 1). In Appendix 1, the numbers of males and females are given together with the year of collection. Sampling has been carried out during the same season each year (September–October), hence minimizing the potential bias in asymmetry estimates because of longer exposure to predators that could have disproportionately killed more asymmetric prey.

Skulls were prepared using dermestid beetles. Positions of 20 landmarks were captured in three dimensions on the ventral side with the Reflex microscope (precision to 0.001 mm; Reflex Measurement, Cambridge, UK). Two landmarks were unpaired, lying on the median plane, and the remaining 18 landmarks were paired (Fig. 2). Each skull was measured three times during independent sessions to control for measurement error. Individuals younger than 1 month, i.e. those corresponding to the age class 1 according to Lidicker (1966), were discarded in order to avoid additional ontogenetic variation in FA.

Fig. 1 (a) The course of the *musculus/domesticus* hybrid zone in Europe. The shaded area shows the present transect. (b) Location of sampling sites. Locality numbers correspond to those in Table 1. The thick dashed line depicts the position of the zone estimated from six autosomal allozyme loci (Macholán *et al.*, 2007; S.J.E. Baird & M. Macholán, unpublished).

Each individual was genetically characterized by a hybrid index (HI) defined as the frequency of diagnostic *musculus* alleles averaged across six diagnostic allozyme loci (*Es1*, *Gpd1*, *Idh1*, *Mpi*, *Np* and *Sod1*). In addition, HI was computed for each locality: for this purpose, larger samples ranging from 10 to 140 specimens per locality were used (see Macholán *et al.*, 2007). Based on their HIs, all localities were then grouped into five introgression classes, denoted as pure *domesticus* (PD: HI = 0.0000–0.1249, $N = 69$), introgressed *domesticus* (ID: HI = 0.1250–0.3749, $N = 67$), hybrid (HY: HI = 0.3750–0.6249, $N = 25$), introgressed *musculus* (IM: HI = 0.6250–0.8749, $N = 54$) and pure *musculus* (PM: HI = 0.8750–1.0000, $N = 73$).

Geometric morphometric procedures

Prior to all other analyses, centroid size was computed for each specimen. This corresponds to the square root of the sum of squared distances between each landmark and the centroid of the landmark configuration (Bookstein, 1991). As shape is defined as all geometric information about an object apart from its size, position and orientation, we adjusted the landmark configurations using the Procrustes superimposition (Rohlf & Slice, 1990). During this procedure, some degrees of freedom are lost: 1 by rescaling (common size), 3 by translation (common x, y, z coordinates of centroids) and 3 by rotation (specific orientation in three dimensions).

Vertebrate skulls are structures with object symmetry (Mardia *et al.*, 2000). This term refers to a kind of symmetry where right and left sides are delimited by an internal median axis or plane so that, in the absence of any asymmetry, they represent mirror images of each other. Thus there are two types of landmarks on the skull: unpaired (lying on the median plane) and paired (lying outside it) and we need to analyse not only the shape of the right and left sides but also how the sides are

Table 1 List of localities sampled, number of specimens analysed (*N*), values of hybrid index (HI), distances along transect, introgression classes based on their HIs and mean values of FA for each population.

Locality	*N*	Hybrid index	Distance along transect (km)	Introgression class	Mean FA
Straas 1	16	0.01	0.00	PD	0.19
Straas 2	34	0.01	0.11	PD	0.21
Benk	12	0.03	7.44	PD	0.19
Aš	7	0.01	29.57	PD	0.15
Plesná	15	0.21	38.56	ID	0.20
Lužná	16	0.21	39.51	ID	0.21
Starý Rybník	4	0.28	41.50	ID	0.17
Dlouhé Mosty	15	0.29	44.56	ID	0.23
Svatý Kříž	8	0.31	44.93	ID	0.18
Dolnice	9	0.22	45.55	ID	0.18
Doubí	17	0.40	45.93	HY	0.23
Jindřichov	8	0.60	46.56	HY	0.22
Milhostov	10	0.68	47.91	IM	0.19
Nebanice	8	0.79	51.22	IM	0.22
Kaceřov 1	7	0.87	52.13	IM	0.24
Kaceřov 2	29	0.81	52.21	IM	0.23
Staré Sedlo	14	0.99	65.42	PM	0.19
Počerny 2	8	0.99	69.83	PM	0.19
Horní Slavkov	7	1.00	71.64	PM	0.19
Nová Ves 1	5	0.99	72.68	PM	0.18
Nová Ves 2	22	1.00	72.80	PM	0.25
Buškovice	17	1.00	108.51	PM	0.22

Introgression classes are delimited as follows: PD, pure *domesticus* (HI = 0.0000–0.1249); ID, introgressed *domesticus* (HI = 0.1250–0.3749); HY, hybrid (HI = 0.3750–0.6249); IM, introgressed *musculus* (HI = 0.6250–0.8749); PM, pure *musculus* (HI = 0.8750–1.0000). The distances are measured from the westernmost locality in direction perpendicular to the centre of the hybrid zone defined with six diagnostic allozyme loci (see Macholán *et al.*, 2007 for details).

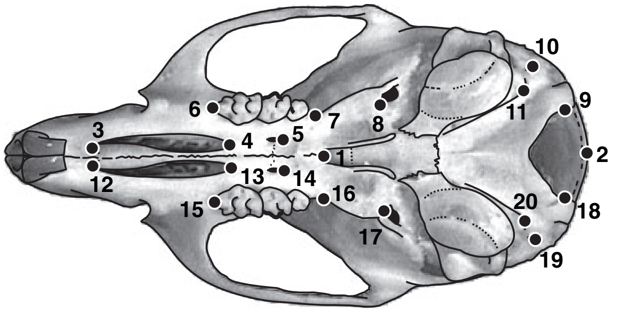

Fig. 2 Definitions of landmarks: 1 – juxtaposition of the palatine bones on their caudal edges; 2 – caudal end of the occipital foramen in the midline (opisthion); 3 and 12 – rostral extremity of the palatine fissure; 4 and 13 – caudal extremity of the palatine fissure; 5 and 14 – caudal extremity of the greater palatine foramen; 6 and 15 – rostral edge of the first molar; 7 and 16 – connection of the palatine bones with the infratemporal crest of basisphenoid bone; 8 and 17 – rostral end of the alar foramen on the wing of basisphenoid bone; 9 and 18 – rostral end of the medial edge of the occipital condyle; 10 and 19 – tip of the paracondylar process, 11 and 20 – crease of the mastoid process of temporal bone.

joined together including local deformations of the median plane. Therefore, mirror configurations were first created for each of the three replicated measurements of each individual, and Procrustes superimposition was carried out simultaneously in the whole data set. After the superimposition, resulting Procrustes coordinates were averaged across all three replicates.

However, in the current geometric morphometric framework, there is no natural measure of size differences between left and right halves of structures with object symmetry (Klingenberg *et al.*, 2002). Therefore, we evaluated only shape asymmetry captured by differences in Procrustes coordinates.

Significance of variability components

On the whole data set, we tested whether DA was different from zero (i.e. significantly higher than FA) and whether FA was significantly higher than measurement error. We applied ANOVA/MANOVA procedures with two-factor design common in FA studies (Palmer & Strobeck, 1986), where individual is the random factor and reflection is the fixed factor. Significant DA and FA are then revealed by the significant effects of reflection and interaction terms, respectively.

Relative magnitudes of DA, FA and measurement error were quantified using a modified ANOVA procedure (Klingenberg & McIntyre, 1998; Klingenberg *et al.*, 2002) where sums of squares were summed over all coordinates and divided by the respective degrees of freedom (see Table 1 in Klingenberg *et al.*, 2002) to obtain appropriate variance estimators. However, modified

MANOVA appeared to be more appropriate to assess the significance of DA and FA, as the null hypothesis of isotropic variation of Procrustes coordinates was rejected ($P < 0.0001$) in the standard test of sphericity applied to nonzero eigenvalues (Mardia *et al.*, 1979).

Modifications of the MANOVA procedure dealing with lack of degrees of freedom due to Procrustes superimposition and with the special nature of data for structures with object symmetry were introduced and fully explained by Klingenberg *et al.* (2002), so we summarize them only briefly here. First, all sums of squares and cross products (SSCP) matrices used in the tests have to describe shape variation of the same dimensionality (which equals to 3 × number of pairs of landmarks + number of unpaired landmarks − 3 d.f. lost due to superimposition, i.e. 26 in this case). Therefore, reflection was tested against the interaction term as usual but inter-individual variation was not tested and individual × reflection interaction was compared with only the asymmetric component of measurement error (estimated as variation of differences between corresponding mirror images across replicated measurements). Second, all SSCP matrices used are singular because their rank is lower than the number of variables. The rank is equal to the lower of two values: dimensionality or the number of degrees of freedom [which is 1 for the reflection, $N - 1$ for the interaction and $N\ (r–1)$ for the asymmetric component of measurement error, where $r = 3$, i.e. the number of replicated measurements]. For each effect, Wilks' lambda was therefore calculated using products of nonzero eigenvalues instead of determinants (Seber, 1984, p. 40). Its significance was assessed by a permutation test based on the randomization of data only with respect to the effect tested. Thus, the reflection term was tested by random flipping of corresponding mirror images and interaction term by random reshuffling of residual configurations remaining after subtraction of individual and reflection means (Klingenberg & McIntyre, 1998). All permutation tests in this study used 10 000 random permutations. *F*-distribution approximations of Wilks' lambda and associated *P*-values were also calculated (Seber, 1984, p. 41).

Individual-signed asymmetries

The mean of mirror configurations is by definition perfectly symmetric. Signed asymmetry of each individual was therefore assessed by subtraction of this mean from the original (nonreflected) configuration. As the shape is inherently a multivariate character, its asymmetry is fully described only by a vector containing left–right deviations of all landmarks in all directions. It should be remembered that asymmetry of every individual is described by a vector with 60 elements, yet with only 26 d.f. (see above).

First, we tested normality of the distribution of individual-signed asymmetries, using Mardia's multivariate measures of skewness and kurtosis (Mardia, 1970). If DI is uniform throughout the population and all left–right differences are of entirely random origin, signed asymmetries should be symmetrically distributed around zero. The expected distribution can be normal (Palmer, 1994; Van Dongen, 1998) or leptokurtic (Graham *et al.*, 2003b; Babbitt, 2006) depending on the particular developmental model. In any case, the distribution shape is of interest because deviations from normality may also indicate the presence of other types of asymmetry or of mixed distributions (Palmer & Strobeck, 1992, 2003; Van Dongen *et al.*, 1999a,b). Moreover, three-dimensional (3D) plots of landmark-specific asymmetries were visually inspected when they showed remarkable clustering of points indicating bimodality (Debat *et al.*, 2000).

To examine size-dependence of asymmetry, multivariate regression of signed asymmetries on natural logarithms of centroid size was performed. The sum of squared covariances of independent and dependent variables was used as a statistic in the permutation test and a marginally significant relationship was found ($P = 0.041$). Residuals from the regression were therefore used as input in further analyses. However, as there may be still some dependence of the magnitude of individual asymmetry on size, we used a test proposed by C.P. Klingenberg (pers. comm.): the magnitude was expressed as a square root of the sum of squared elements of the asymmetry vector (i.e. Procrustes distance between mean of mirror configurations and nonreflected configuration), and natural logarithms of the Procrustes distances were then regressed on natural logarithms of centroid size. If the regression is significant, individual asymmetry vectors are divided by antilogarithms of residuals from the regression. In the present study, the result was not significant ($P = 0.251$) and thus the correction was unnecessary.

Similarly, influence of sex was examined by MANOVA of signed asymmetries and ANOVA of corresponding Procrustes distances but in neither case was the result significant (Wilks' lambda = 0.927, $P = 0.748$ in MANOVA and $F = 0.293$, $P = 0.460$ in ANOVA). Both sexes were therefore pooled.

Comparison of asymmetries

Our data are hierarchically organized, individuals belong to localities and localities belong to introgression classes. Signed asymmetries were therefore compared in a hierarchical manner, two questions being posed. First, are mean asymmetries significantly different between the introgression classes relative to differences between localities within each of them? Second, are mean asymmetries between localities within the same class significantly different relative to individual differences within them? Significant differences indicate significant differences in DA at that particular level. Nested effect (localities within classes) was tested with MANOVA. Wilks' lambda was used as a test statistic and significance was assessed through an *F*-approximation as well as by a permutation test where individual asymmetries were randomly reshuffled. However, the main effect (classes) was tested with ANOVA (sums of squares added up across all variables) because the nested effect had less degrees of freedom than was the dimensionality of asymmetric shape subspace, precluding the use of a multivariate procedure. Proportions of variance explained on different levels were also calculated by means of summing sums of squares across all variables.

Prior to analyses of FA, locality means were subtracted from individual-signed asymmetries. After this correction for DA, overall magnitudes of individual asymmetries were expressed as Procrustes distances. They were compared with ANOVA in the same hierarchical manner as signed asymmetries: classes vs. localities within them and localities within classes vs. individuals, significant results indicating differences in FA; *P*-values were estimated parametrically as well as with permutation tests with random reshuffling of Procrustes distances. Finally, a polynomial model was fitted to describe the relation of individual unsigned asymmetries (Procrustes distances) to individual HIs.

In addition, we included the year of capture as a categorical predictor nested within locality. Thus, differences between localities belonging to the same class were tested against differences between mice captured in different years at the same site. Only sufficient and balanced samples collected over several years were included in this analysis. This reduced data set comprised 170 specimens from 10 localities: Straas 1, Straas 2, Benk, Lužná, Dlouhé Mosty, Doubí, Milhostov, Kaceřov 2, Staré Sedlo and Buškovice. Significance of all effects was tested by separate ANOVAS for DA and FA. In comparison of DAs, Procrustes ANOVA was used, i.e. sums of squares were summed up across all variables and divided by appropriate degrees of freedom (Klingenberg & McIntyre, 1998).

Patterns of shape variation

Theoretically, FA could be symmetrically distributed around some mean value and reflect passive developmental noise. In such a case, variation between group means (DAs) should be entirely independent of variation within groups (which is in fact FA, because the level of population FA generally corresponds to the variance of individual asymmetries). There should be therefore no similarity between within-locality and locality-within-class covariance matrices. On the other hand, if DAs of differently delimited groups have the same nature, patterns of their variation should be congruent. Thus, similarity of between-class and locality-within-class covariance matrices would be expected.

Similarity of matrices was quantified as Pearson correlation between them and tested with the Mantel test. The procedure was, however, modified for shape variables of a structure with object symmetry according to Klingenberg *et al.* (2002). Only paired landmarks from one side were used for computation of covariance matrices. Variances and covariances of three coordinates of the same landmark were excluded from the calculation of matrix correlation and in the Mantel test, rows and columns corresponding to these coordinates were reshuffled jointly.

Software

Centroid size and all shape variables were extracted using PAST, ver. 1.55 (Hammer *et al.*, 2001). Statistical tests and computations were performed with Statistica, ver. 7.1 (StatSoft, Inc., 2006) and MATLAB, ver. 6.5 (The MathWorks, Inc., 2004).

Results

Significance of DA and FA, and the distribution of asymmetries

Results of MANOVA of Procrustes coordinates are summarized in Table 2 together with proportions of total variation explained by each factor revealed by Procrustes ANOVA. Both a reflection effect and an individual × reflection interaction were found to be highly significant ($P < 0.001$), explaining small or moderate proportions of total variation (reflection: 0.42%, interaction: 8.31%). Individual effect, tested through Procrustes ANOVA, was also highly significant ($P < 0.001$) explaining 87.26% of total variation. Results of parametric and permutation tests were very similar so that only *P*-values are presented.

The asymmetric component of measurement error accounted for 10.49% of FA. Our choice of triple measurements seem to be adequate to this relative amount of measurement error (Van Dongen, 1999), and our estimates of individual asymmetries should not

Table 2 Results of analyses of variance of Procrustes coordinates.

Effect	Wilks' lambda	*F*	*P*	d.f.	% explained
Individual	–	10.11	< 0.0001	7749	87.26
Reflection	0.34	19.75	< 0.0001	26	0.42
Individual × reflection interaction	2.81×10^{-22}	11.30	<0 .0001	7462	8.31
Measurement error				30 528	4.02

Wilks' Lambda of each effect was approximated by *F*-ratio according to Seber (1984) whereas permutation tests were used for computing *P*-values. In the case of individual effect, *F*- and *P*-values, degrees of freedom and the proportion of total variance explained were acquired through Procrustes ANOVA.

be substantially biased, although they comprise both FA and measurement error, as averaging of three measurements decreases ME to one-third (ca 3.5% of the FA).

Mardia's tests of multivariate normality revealed that multivariate signed asymmetries were significantly skewed ($V_{3276} = 55\,405$, $P < 0.001$) and leptokurtic ($U = 768.17$, $P < 0.001$). No sign of platykurtosis, indicating antisymmetry, was found. This was corroborated by the lack of remarkable clustering of individual points around each landmark in 3D plots of landmark-specific asymmetries (not shown).

Differences in DA and FA across the hybrid zone

Results of comparisons of directional and fluctuating asymmetries are summarized in Tables 3 and 4, respectively. Only *P*-values from permutation tests are presented but parametric tests usually yielded very similar results. For both DA and FA, the nested effect was significant but the main effect was not. In other words, differences between introgression classes were not sufficiently large relative to differences between localities belonging to them but the within-class differences were much larger than differences between individual-signed asymmetries within localities. Introgression classes explained 1.92% of total variation in DA and 4.00% in FA, these proportions being much higher for locality-within-class effect (6.64% for DA and 14.47% for FA). Individual unsigned asymmetries (Procrustes distances) were best fitted to individual HIs by a second-order polynomial (Fig. 3), and the regression was significant ($F = 6.88$, $P = 0.002$), despite explaining only 4.60% of the variation. The highest unsigned asymmetry was predicted by the regression for HI = 0.654.

When year was included as an additional nested factor, all effects became nonsignificant for both DA and FA (Tables 5 and 6). Correlation of between-class and locality-within-class covariance matrices (and hence of variation of corresponding DAs), was 0.340 ($P = 0.020$). This was, however, as high as 0.609 ($P < 0.001$) between covariance matrices of locality-within-class mean asymmetries (DAs) and within-locality individual asymmetries (FAs).

Table 3 Results of analyses of variance of signed asymmetries.

Effect	Wilks' Lambda	*F*	*P*	d.f.	% explained
Introgression classes	–	1.27	0.18	4	1.92
Localities within classes	0.13	1.21	< 0.01	17	6.64
Individuals within localities				266	91.44

Wilks' lambda, *F*-ratio, *P*-value, degrees of freedom and proportion of variance explained are presented for each source of variation. *F*-ratio was approximated according to Seber (1984) and *P*-values were obtained using permutation tests. Differences between introgression classes were tested using ANOVA whereas MANOVA was used for all other tests.

Table 4 ANOVA of unsigned asymmetries (Procrustes distances).

Effect	*F*	*P*	d.f.	% explained
Introgression classes	1.17	0.36	4	4.00
Localities within classes	2.78	< 0.001	17	14.47
Individuals within localities within classes			266	81.53

F-ratio, *P*-value from permutation test, number of degrees of freedom and proportion of variance explained are presented for each source of variation.

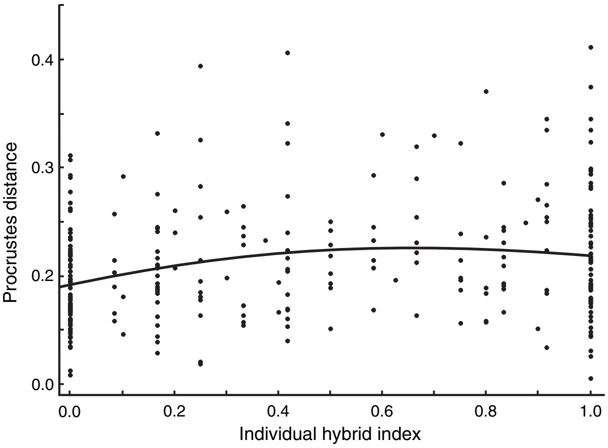

Fig. 3 Individual Procrustes distances plotted against individual hybrid indices based on six diagnostic autosomal loci. The regression equation is $y = 0.19 + 0.10x - 0.08x^2$.

Discussion

FA as a measure of DI

As mentioned above, asymmetries arising due to internal stochastic fluctuations correspond exactly to FA only under very specific conditions. First, no genetic or epigenetic variability for left–right asymmetry may be present in a population. Even if mean asymmetry is equal to zero, left–right differences may be partly deterministic (Palmer & Strobeck, 1992). Second, DI must be the same on both sides of the body (Klingenberg, 2003). And finally, microenvironment must be exactly identical for both sides of each individual (Nijhout & Davidowitz, 2003).

In this study, some idealization is likely to be introduced by using FA as a measure of DI. Most importantly,

Table 5 Procrustes ANOVA of signed asymmetries with year of capture as an additional nested factor.

Effect	*F*	*P*	d.f.	% explained
Introgression classes	0.97	0.51	104	3.10
Localities within classes	1.19	0.34	130	3.97
Years within localities within classes	1.17	0.30	468	11.99
Individuals within years within localities within classes			3692	80.93

F-ratio, *P*-value from permutation test, number of degrees of freedom and proportion of variance explained are presented for each source of variation.

Table 6 ANOVA of unsigned asymmetries (Procrustes distances) with year of capture as an additional nested factor.

Effect	*F*	*P*	d.f.	% explained
Introgression classes	0.97	0.49	4	4.50
Localities within classes	1.75	0.18	5	5.82
Years within localities within classes	1.22	0.26	18	12.01
Individuals within years within localities within classes			142	77.67

F-ratio, *P*-value from permutation test, number of degrees of freedom and proportion of variance explained are presented for each source of variation.

we have no knowledge about the amount of genetic and/or epigenetic variation for the deterministic break of left–right symmetry. However, it may be present as we found mean signed asymmetry to be significantly different among localities, at least when year-dependent differences were ignored. Furthermore, we have no information about the microenvironmental variation, yet this cannot be ruled out as it can be brought about, for example, by different positions of individual embryos in the uterus (Nijhout & Davidowitz, 2003). Moreover, even if all asymmetries are of purely random origin, they do not reflect DI reliably. As discussed by Whitlock (1996, 1998) and Van Dongen (1998, 2001), FA is inherently a poor estimator of DI as it estimates within-individual developmental variance with only 1 d.f. These authors introduced methods for assessing variation of DI from the level of FA (reviewed in Van Dongen, 2006) but their application is problematic in our case. These methods were developed for usual univariate size differences of separate structures and their reliability is limited by the validity of the developmental model used. Shape is, however, a multivariate trait and development of skull symmetry is likely to be complex. Fortunately, the impact of a discrepancy between FA and DI is less severe when population FA is studied (Whitlock, 1996).

Another idealization may have been introduced by our nested design of data processing. First, mice trapped at particular sites may not be genetically homogenous, especially in the centre of the zone, so that computing mean population HIs and delimiting the introgression classes according to these HIs (cf. Table 1) may introduce an error. Nevertheless, when introgression classes were based on individual rather than population HIs, the analyses yielded very similar results (not shown). Second, the introgression classes were considered as homogenous and thus differences among localities within the same class were assumed to be introgression-independent, although individual populations certainly differed in HIs. However, substantial variation was found even between localities with almost the same HIs, and this bias therefore should not be very important.

Frequency distribution of asymmetries

Multivariate distribution of signed asymmetries was significantly leptokurtic and skewed in this study. There are two principal, not mutually exclusive, explanations for departures of asymmetry distributions from normality. First, we may see a mixture of different normal distributions (Palmer & Strobeck, 1992). When these normal distributions have different variances, their mixture is leptokurtic. When they have different means, the mixture is platykurtic. Differences in both parameters can lead to various patterns including skewness. The second explanation is based on the idea that normal distribution of asymmetries is expected only under a specific developmental model (Graham *et al.*, 2003b; Babbitt, 2006). For example, when growth is multiplicative rather than additive, the leptokurtic distribution and size scaling of asymmetries is expected and the pattern can be further complicated by a regulatory feedback between sides. Babbitt *et al.* (2006) found that the distribution of asymmetries in three insect species fits better to the double Pareto lognormal distribution implying that the growth of structures may be better described by geometric (multiplicative) rather than ordinary (additive) Brownian motion. Moreover, there may be a regulatory feedback between sides, yet the evidence is equivocal (Swaddle & Witter, 1997; Aparicio, 1998; Van Dongen *et al.*, 1999a,b; Kellner & Alford, 2003; Møller & Van Dongen, 2003; Petavy *et al.*, 2006; Stige *et al.*, 2006). Thus we cannot use any simple growth model to test explicitly whether it is sufficient to explain the observed shape of the distribution.

DI and hybridization

There are conflicting expectations about the impact of hybridization on DI. It was reasoned that hybridization could lead both to the increase or decrease of DI (and hence FA): decrease of DI can be caused by higher heterozygosity of hybrids whereas increase of DI results from a disruption of differentially coadapted gene complexes (Graham, 1992; Clarke, 1993). A comprehensive review by Alibert & Auffray (2003) seems to confirm predictions of these hypotheses. Increased FA was observed more frequently in hybrids between distant taxa, where the occurrence of substantially different genomic coadaptation is more likely, whereas decreased FA was more often found in hybrids between closely related taxa, where the positive impact of increased heterozygosity should predominate. On the other hand, this correspondence was only loose and levels of FA often differed between measured traits. This heterogeneity would not be surprising if the impact of hybridization on DI depended on special properties of differences between maternal populations. In such instance, overall genomic measures such as heterozygosity or genetic distance may be unable to predict either its direction or magnitude. Moreover, dependence of FA on heterozygosity and disruption of genomic coadaptation were stated only on the grounds of correlation studies, not by insight into developmental processes underlying FA in actual populations (Alibert & Auffray, 2003; Woolf & Markow, 2003; but see Rego *et al.*, 2006). Finally, hybridization can not only destabilize the development but also generate new variation for individual asymmetry, contributing to FA without the change of mean asymmetry and leading to overestimates of DI. This possibility was only recently taken into account and shown to explain increased FA in *Drosophila* hybrids (Rego *et al.*, 2006).

DI in house mouse hybrids

FA has been studied in two types of house mouse hybrids. The first type was hybrids between chromosomal races of *M. m. domesticus* characterized by different combinations of Robertsonian fusions (see Piálek *et al.*, 2005 for a review of chromosomal variation in the house mouse). The results seem to be case-dependent, and in some cases, genic rather than chromosomal differentiation is likely to be responsible for the observed patterns (Chatti *et al.*, 1999; Auffray *et al.*, 2001; Gazave *et al.*, 2006; Muñoz-Muñoz *et al.*, 2006). The second class was *musculus/domesticus* hybrids in which only decreased FA has been reported so far. In the Danish portion of the mouse hybrid zone, Alibert *et al.* (1994) found decreasing FA in lower molars towards the zone centre and similar pattern was found also in comparison with random-bred laboratory strains derived from populations from the edges of the hybrid zone in Denmark and their hybrids. Alibert *et al.* (1997) crossed these strains to produce several classes of offspring (individuals of either of pure strains and F1, F2 and various types of BC hybrids). FA in hybrid classes was again generally lower than in pure parental classes but there was no difference among various types of hybrids. However, *musculus* individuals were more asymmetrical than *domesticus* ones, the pattern not observed in the original field study. In the same strains and their F1 hybrids, Debat *et al.* (2000) measured FA of 2D projections of the dorsal side of the skull and they also found decreased FA in hybrids. The results of these studies were interpreted as evidence of a heterotic effect due to higher heterozygosity in hybrids. As the decrease of FA was observed already in F1 hybrids with non-recombined genomes and did not change significantly in subsequent crosses, recombination appeared to play a minor role, if any, in changing the level of FA. Finally, no correlation between FA and fitness was found (Alibert *et al.*, 1997).

DI along the central-European transect

As shown in Tables 3 and 4, highly significant differences in FA were found between localities belonging to the same introgression class. When year was included as an additional factor the differences became nonsignificant (Tables 5 and 6); however, it is not clear to what extent this result is caused by the decrease of statistic power owing to adding a further parameter in the analysis.

There may be various environmental and genetic stressors responsible for the variation in FA. In rodents, FA has been shown to increase, at least in some cases, due to contamination (Nunes *et al.*, 2001; Oleksyk *et al.*, 2004; Veličković, 2004), landscape fragmentation (Marchand *et al.*, 2003), specific mutations (Willmore *et al.*, 2006) and chromosomal aberrations (Leamy *et al.*, 2001). Although all these factors are likely to affect DI (and hence FA), our data do not allow us to assess their importance. What we can conclude, however, is that introgression class explained much less variation than locality and/or year in our data. This suggests that the impact of hybridization on FA is either negligible or much lower than the influence of other factors and hence masked by more substantial variation due to environmental and/or genetic stressors not related to inter-subspecific hybridization.

When differences between introgression classes were not controlled for within-class variation (results not presented), *domesticus*-like mice (classes PD, ID) revealed significantly lower level of FA than *musculus*-like mice (classes PM, IM), in agreement with results of Alibert *et al.* (1997). However, FA in the HY class was higher (although nonsignificantly) than in both *musculus*-like classes; in other words, contrary to Alibert *et al.* (1994, 1997) and Debat *et al.* (2000), we found no evidence of decreased DI in hybrids from the central-European portion of the house mouse hybrid zone. This is clearly illustrated by the significant quadratic polynomial function fitted to individual asymmetries plotted against individual HIs (Fig. 3), predicting the highest FA for HI ≈ 0.65.

This discrepancy can be explained in three ways. First, we can hypothesize that mice from the zone centre suffered from severe environmental stress and/or unfavourable social conditions resulting, for instance, from local population outbreaks. Although differences in the local environment seem improbable in this commensal species, we cannot rule out effects of some unpredictable conditions such as presence (and consumption) of poisonous substances, corn treated with pesticides, etc., potentially reducing health (and thereby increasing DI) of the animals. More importantly, several cases of an extreme local outbreak resulting from the presence of spatially limited favourable conditions were recorded during the sampling campaigns, leading to decreased fitness manifested through lower fecundity, higher parasitic load, etc. (J. Piálek & M. Macholán, unpublished). Higher FA in hybrids then can be merely a result of these factors.

Second, different traits may display different patterns of FA (Polak *et al.*, 2003) even in such an integrated structure as the mouse skull as suggested by Rohlf & Corti (2000) and Macholán *et al.* (2007) who found different patterns of morphological change in the dorsal and ventral parts of the skull. Finally, there may be differences in the history, structure and dynamics of the hybrid zone between Denmark and central Europe. The zone is assumed to be much older in southern and central parts of Europe than in northern parts of the continent (Auffray *et al.*, 1990; Cucchi *et al.*, 2005; see also discussion in Macholán *et al.*, 2007), and hence there could have been more time for homogenization of divergent gene pools by spread of neutral alleles (Endler, 1977; Barton & Hewitt, 1985). There may be also differences in finer-scale geographic structure of the zone leading, for example, to different dynamics of extinction-recolonization events and gene flow.

The DA analysis revealed results similar to the FA analysis: among-class differences were not significant relative to differences among localities belonging to them whereas the latter were significantly different relative to the variation of individual-signed asymmetries. The congruence of DA and FA patterns is also suggested by the highly significant correlation between within-locality and locality-within-class covariance matrices (correlation coefficient = 0.6086). The simplest explanation of this observation is the skewness of the distribution of asymmetries. There may also be a link between the extent of deterministic breaking of symmetry and DI. More asymmetric structures may be inherently more instable or a shift of DA itself may result from the interplay between deterministic and stochastic developmental processes as discussed by Graham *et al.* (2003a) and demonstrated by Kark (2001). Alternatively, there may be inherent variation for asymmetry, segregating in populations and thus contributing to differences in means as well as variances of asymmetries.

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Appendix 1

List of sampling sites with year of collection and the number of males (M) and females (F) trapped

Straas 1: 2001 – 1M,4F; 2002 – 5M,3F; 2003 – 2M,1F; Straas 2: 2000 – 3M,4F; 2001 – 1M,4F; 2002 – 4M,10F; 2003 – 4M,4F; Benk: 2002 – 4M,3F; 2003 – 5M,0F; Aš: 2001 – 1M,6F; Plesná: 1997 – 8M,5F; 1999 – 1M,1F; Lužná: 1999 – 2M,3F; 2000 – 2M,1F; 2001 – 1M,1F; 2002 – 2M,1F; 2003 – 2M,1F; Starý Rybník: 2001 – 1M,0F; 2002 – 1M,1F; 2003 – 1M,0F; Dlouhé Mosty: 2000 – 2M,2F; 2001 – 5M,6F; Svatý Kříž: 2002 – 5M,2F; 2003 – 0M,1F; Dolnice: 1997 – 4M,5F; Doubí: 2000 – 2M,2F; 2001 – 6M,0F; 2002 – 2M,1F; 2003 – 2M,2F; Jindřichov: 2000 – 3M,5F; Milhostov: 2000 – 2M,5F; 2001 – 1M,2F; Nebanice: 2002 – 3M,5F; Kaceřov 1: 1999 – 3M,3F; 2003 – 1M,0F; Kaceřov 2: 1999 – 3M,2F; 2001 – 3M,13F; 2002 – 2M,0F; 2003 – 3M,3F; Staré Sedlo: 2002 – 3M,6F; 2003 – 4M,1F; Počerny 2: 2002 – 4M,3F; 2003 – 0M,1F; Horní Slavkov: 2003 – 5M,2F; Nová Ves 1: 2003 – 1M,4F; Nová Ves 2: 2001 – 10M,11F; 2002 – 1M,0F; Buškovice: 2000 – 4M,7F; 2003 – 2M,4F.