Can fluctuating asymmetry in morphological traits be used to detect inbreeding in Drosophila ananassae?

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Abstract

Fluctuating asymmetry (FA, non-directional deviations from perfect bilateral symmetry) often has been used as a measure of developmental instability (DI) in populations. It is expected to increase in the populations subjected to genetic stressors such as inbreeding or hybridization and environmental stressors such as toxins or parasites, although the results have not always been consistent. In the present study, we have tested the effect of inbreeding on trait size and fluctuating asymmetry in different morphological traits in seven laboratory strains of *Drosophila ananassae*, which are maintained in the laboratory for several generations under full–sib design. The trait size of sternopleural bristle number, wing length, wing to thorax ratio, sex comb tooth number, and ovariole number varies significantly among the strains. Fluctuating asymmetry of measured traits also differs significantly among the populations in females (except sternopleural bristle number) and in males (except sternopleural bristle number and wing to thorax ratio). Further, the positional fluctuating asymmetry, which is a sensitive measure of DI, varies significantly for sternopleural bristle number and sex comb tooth number. There is also significant difference among the strains and between sexes for the composite fluctuating asymmetry. These results are discussed in relation to the use of FA as an indicator of inbreeding.

Introduction

Developmental instability (DI) refers to the inability of an organism to correct for random accidents under development and has become a major topic in evolutionary biology. The failure to buffer the phenotype against perturbations in bilateral traits can be manifested as fluctuating asymmetry (FA), which is defined as small and random departures of different bilateral traits of an organism from the perfect symmetry (Ludwig, 1932; Van Valen, 1962; Trotta *et al.*, 2005). The underlying assumption of fluctuating asymmetry (FA) is that both sides of a bilateral trait are under the control of the same genes and any deviation from the bilaterally symmetrical phenotype would be due to perturbations of either environmental or genetic in origin during ontogeny (Mpho *et al.*, 2002). Thus, the left-right asymmetry of morphological traits implies the perturbations in developmental homeostasis at the molecular, chromosomal and epigenetic levels (Parsons, 1992). For fluctuating asymmetry to be a useful measure of DI, it is necessary to account for size dependence, measurement error, and possible departures from ideal FA (normally distributed deviations from symmetry with a mean of zero) such as directional asymmetry (from symmetry with a mean different from zero) and antisymmetry (deviations from symmetry with a bimodal distributions; Palmer and Strobeck, 1986, 2003). Many studies have examined how fluctuating asymmetry responds to changes in genetic and environmental parameters, but no clear pattern has been found due to inconsistent results (Waldman, 1999; Leamy and Klingenberg, 2005; Van Dongen, 2006).

Inbreeding, that is the mating of related individuals, promotes homozygosity, which is expected to render organisms less able to cope up with changes in the environment and make them less fit (Lerner, 1954; Leamy *et al*., 2001). The deleterious effects of inbreeding on fitness traits have been reported in many organisms (Roff, 1998). Also, inbred individuals show greater variability than outbred in most characters measured (Falconer and Mackay, 1996; Whitlock and Fowler, 1999), but they do not always exhibit higher levels of FA in these characters (Clarke, 1993; Gilligan *et al*., 2000). Thus, the relationship between FA and inbreeding appears tenuous (Møller and Swaddle, 1997; Vøllestad *et al.,* 1999; Leamy and Klingenberg, 2005).

In view of this, the present study was undertaken in order to test the relationship between inbreeding and fluctuating asymmetry in seven laboratory populations of *Drosophila ananassae*. Our design is different from that of most other studies in three ways. First, the model system that we have used is *D. ananassae,* which is a cosmopolitan and domestic species and occupies a unique status among the *Drosophila* species because of certain unusual genetic features (Singh, 2000). Second, we are using seven inbred stocks of *D. ananassae*, which have spent varying numbers of generations in the laboratory. And third, we are using different morphological traits, *e.g*., wing length, wing to thorax ratio, sex comb tooth number, sternopleural bristle number, and ovariole number in order to test the effect of the inbreeding on size and fluctuating asymmetry. The results of these investigations are reported in this communication. Moreover, previous studies in *D. ananassae* have shown that fluctuating asymmetry exists in controlled laboratory conditions (Vishalakshi and Singh, 2006), there is negative relationship between FA and sexual selection (Vishalakshi and Singh, 2008 a). There is also an effect of different environmental stressors (Vishalakshi and Singh, 2008 b, c) and mutations on the levels of fluctuating asymmetry in *D. ananassae* (Vishalakshi and Singh, 2008 d).

Materials and Methods

Drosophila stocks: In the present study, we have used seven mass culture laboratory stocks of *Drosophila ananassae*, established from naturally impregnated females collected from different geographical localities of India (Table 1). All the stocks were maintained in the laboratory on simple yeast agar culture medium at approximately 24ºC with 60-70% average humidity.

Table 1. Details of the different laboratory stocks of *D. ananassae* used during the study.

Populations	Place of origin	Year of collection
BH	Bhubhaneswar, Orissa	1984
BA	Baripada, Orissa	1987
KO	Kottayam, Kerela	1993
PC	Pondicherry	1999
SK	Shaktinagar, U.P.	2002
PC	Pondicherry	2005
MB	Mumbai, Maharastra	2006

Trait measurements: From each population, five day old males and females (n = 50) were chosen randomly for measuring the different morphological traits, *viz*., thorax length, wing length, wing to thorax ratio, sex comb tooth number, sternopleural bristle number, and ovariole number. Thorax length was measured from the anterior end of the thorax to the posterior end of the scutellum. For wing length, absolute length between the anterior crossvein to the distal tip of the third longitudinal vein was measured under a microscope at 50× magnification using ocular micrometer (1unit = 16.67 µ). The wing to thorax ratio (W/T) ratio was calculated from the data of wing and thorax lengths. On the sternopleurum of males and females, 2 sets of bristles are present. Anterior bristles (A) occur in an oblique row from the forecoxa towards the midline, whereas the transverse bristles (T) running in a thin line toward the center of fly just

anterior to middle leg. The anterior and transverse sternopleural bristles were counted under stereo binocular. Total number of sternopleural bristles was taken as the sum of anterior and transverse bristles. In females, ovaries were dissected out on a glass slide containing a drop of insect's saline with the help of needles under zoom binocular. Ovaries were then transferred on a cleaned slide having a drop of 2% acetocarmine and stained for 2 min and washed and mounted in 45% acetic acid for the proper visualization of ovariole number under a microscope at 50× magnification. Sex combs in males of *D. ananassae* are characterized by several transverse rows of stout blackish bristles on the ventral surface of first, second and third tarsal segments of prothoracic legs. Forelegs of males were dissected and mounted in insect saline, and the number of the teeth on the first (C1), second (C2), and third tarsal segments was counted under a microscope. Total number of sex comb teeth per leg includes the teeth on first (C1), second (C2), and third segments.

Statistical analysis: As measurement of morphological traits showed no significant deviations from normality in the Kolmogorov-Smirov test for goodness of fit, no transformation was used for the traits studied. The framework laid by Palmer (1994) and Palmer and Strobeck (1986, 2003) was followed. Measurement error (ME) can cause biased estimates of FA, and repeat measurements should be undertaken to ensure that FA is detectable and there is no confounding effect associated with directional asymmetry (Palmer, 1994; Woods *et al*., 1999). In order to estimate the measurement error, twenty flies were randomly taken from the culture bottles and two replicate counts were made for different traits per fly, each on different day in different order. The magnitude of ME was assessed by two way mixed model ANOVA, where sides were entered as fixed factor and individuals as a random factor. In all ANOVA (not shown), the interaction between side and individual is highly significant ($P < 0.001$), indicating that the measurement error in all the traits is negligible compared with the variation between sides. For each individual, we have calculated individual asymmetry as D = R-L (where R is the value of the trait on the right side and L is the value of the trait on the left side) and trait size as mean (R+L)/2. For directional asymmetry, one sample t-test on the signed difference (R-L) for each trait and genotype was performed to determine whether the mean (R-L) values differ from zero (Palmer, 1994). For antisymmetry, we checked departures from normality of distribution of the signed differences (R-L) using Kolmogorov –Smirov test. We have also calculated the skewness and kurtosis coefficients for all the morphological traits and determined whether skewness and kurtosis values deviated from zero (which is the expected value for normal distribution) by one-sample t-test (Palmer, 1994). If no DA is present and the distribution if signed differences is normal then the variation in these differences represent classical FA (Palmer, 1994).

Fluctuating asymmetry has been calculated for a given trait as the mean of the absolute value of the difference in trait size between the right and left side of the body, |(R-L)|. Thus, FA was calculated as mean|(R-L)| for sternopleural bristle number, wing length, wing to thorax ratio, ovariole number, and sex comb tooth number. Positional fluctuating asymmetry (PFA) (Polak, 1997), a measure of the difference between the two sides of the body in the way in which components of a meristic trait are arranged or positioned, was calculated for both sternopleural bristle and sex comb tooth number (Polak *et al.,* 2004). For the sternopleural bristle number, positional fluctuating asymmetry (PFA_B) was calculated as |(Right A /Right T) – (Left A /Left T)| and for sex comb tooth number (PFA_S) as |(Right C1 /Right C2) – (Left C1 /Left C2)|. Trait size for each trait was measured by the average value of right and left side (R+L)/2. Further, to test size dependence between absolute trait asymmetry |(R-L)| and trait size (R+L)/2, we obtained non-parametric Spearman's correlation rank test for all the traits in males and females as suggested by Palmer (1994).

To test whether trait size differed among populations, one-way ANOVA was performed for all traits in males and females. To detect the variation in the levels of FA among different strains for

each trait, we have employed one-way ANOVA. The value of fluctuating asymmetry across the multiple traits was calculated by pooling all the information of multiple traits in composite fluctuating asymmetry analyses (Leung *et al.,* 2000). CFA is calculated as summation of absolute FA values across traits (j) for each individual (i) and then compared by single factor analysis of variance for both populations and sexes {CFA_i= | FA_{ij} | j = 1 to k; (k represents the number of traits per individual)}.

Table 2. Mean ± SE of trait size of different morphological traits among different laboratory stocks of *D.ananassae.*

Traits	Sex	BH-84	BA-87	KO-93	PC-99	SK-02	PC-05	MB-06	$F_{6, 343}$
SBN	M	8.10	8.07	7.94	7.97	8.08	8.09	7.90	0.220
		±0.86	±0.07	±0.97	±0.398	±0.09	±0.123	±0.104	
	F	8.27	7.97	8.06	8.27	8.43	9.08	8.48	2.778*
		±0.97	±0.083	±0.865	±0.125	±0.112	±0.520	±0.104	
WL	M	71.35	71.44	74.35	65.94	71.92	71.32	72.30	34.38**
		±0.513	±0.741	±0.257	±0.312	±0.500	±0.300	±0.242	
	F	84.76	81.56	84.02	74.69	82.97	81.60	82.49	70.582**
		±0.171	±0.388	±0.258	±0.664	±0.393	±0.326	±0.243	
W/T	M	1.33	1.322	1.372	1.337	1.300	1.301	1.30	6.596**
		±0.009	±0.015	±0.008	±0.008	±0.014	±0.007	±0.008	
	F	1.350	1.353	1.365	1.374	1.345	1.344	1.335	2.590*
		±0.0052	±0.0078	±0.006	±0.014	±0.0086	±0.008	±0.0066	
ST	M	35.06	36.32	33.08	28.88	43.07	33.42	35.96	99.25**
		±0.487	±0.348	±0.379	±0.399	±0.560	±0.423	±0.394	
	F	12.09	12.13	12.53	12.60	13.57	12.85	12.23	4.891**
		±0.190	±0.023	±0.227	±0.236	±0.260	±0.273	±0.224	

*P<0.01; **P<0.001
SBN: Sternopleural bristle number; WL: Wing length; W/T: Ratio of wing length and thorax length; ON: Ovariole number; SCTN: Sex-comb tooth number; M: Males; F: Females.

Results

The laboratory populations vary significantly for the trait size of different morphological traits, *viz*., sternopleural bristle number (SBN), wing length (WL), wing to thorax (W/T) ratio, sex comb tooth number (SCTN), and ovariole number (ON) varies significantly in females and in males (except SBN) (Table 2). Descriptive statistics of signed (R-L) asymmetry values for each trait and sex are shown in Table 3. One sample t-test revealed that mean value of each trait did not differ significantly from zero ($P > 0.05$) for all the traits in females and males. Also, none of the (R-L) frequency distributions showed bimodal distributions (Figure 1), and the distributions of the signed differences (R-L) showed normal distribution in the Kolmogorov Smirov test for normality. Moreover, the skewness and kurtosis values did not differ from zero ($P > 0.05$) for any trait (Table 3).

Absolute trait asymmetry (|R-L|) was correlated with trait size (R+L)/2. The significant positive correlation was found in males, for SBN ($r = 0.179$, $P < 0.001$), WL ($r = 0.184$, $P < 0.001$), W/T ratio ($r = 0.162$, $P < 0.001$) and PFA_B ($r = 0.250$, $P = 0.001$); however, SCTN ($r = 0.023$, $P = 0.667$) and PFA_S ($r = 0.043$, $P = 0.427$) have shown no dependence. In females, positive correlation

was found for SBN (r = 0.069, P = 0.201), WL (r = 0.064, P = 0.230), ON (r = 0.127, P = 0.017) and PFA_B (r = 0.300, P = 0.0001) and negative correlation for W/T (r = -0.033, P = 0.533). Thus, where significant correlation existed between FA and trait size, the data of FA were corrected as relative index of FA. No significant correlation was found between relative index of FA and trait size in any traits studied after correction (analysis not shown).

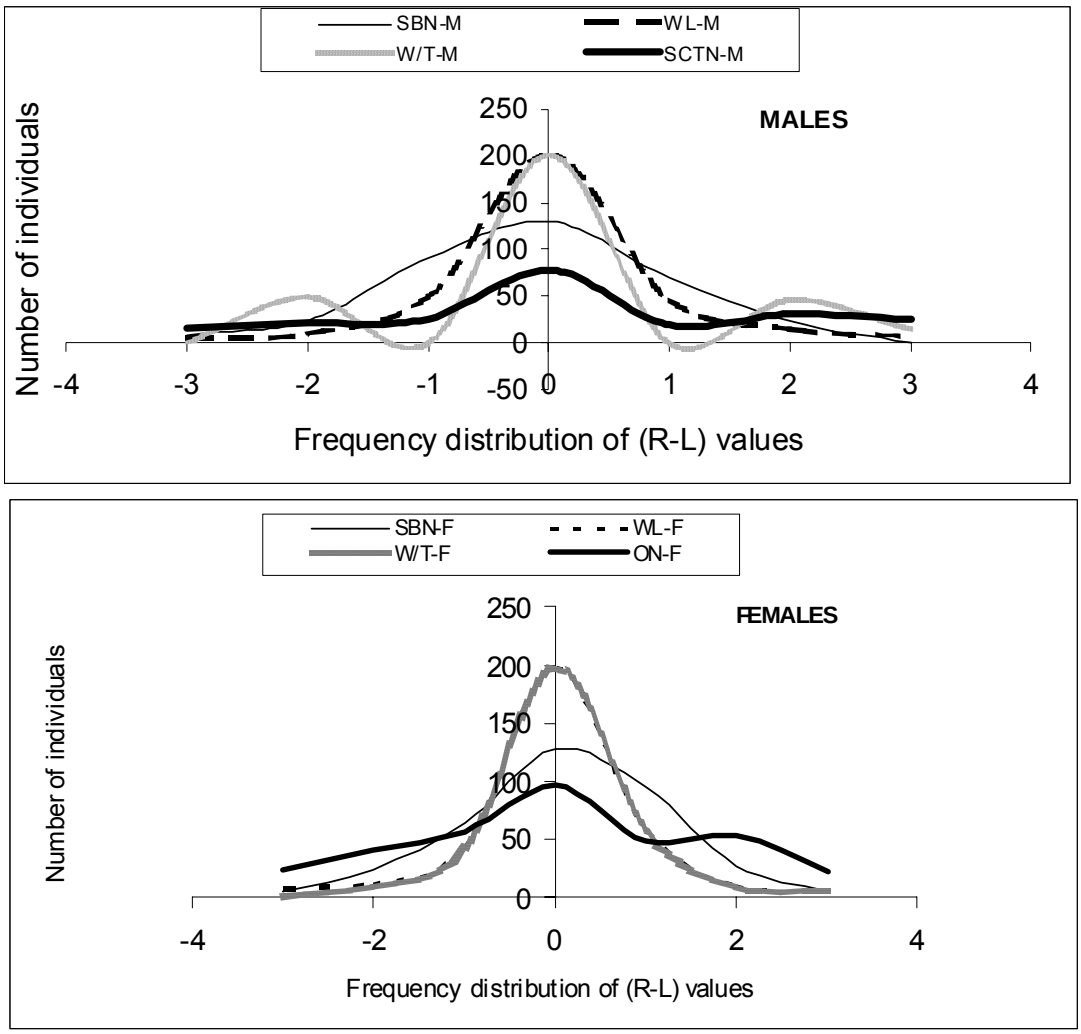


Figure 1. Frequency distribution of the (R_i-L_i) values over the proportion for males and females for all traits. (The abbreviations used: SBN -Sternopleural bristle number, WL- Wing length, W/T-Ratio of wing length and thorax length, SCTN-Sex comb teeth number, ON- Ovariole number).

From Figure 2, it is evident that there are differences in the relative FA values in seven-laboratory populations for all the morphological traits in males and females. To test this statistically, we have employed one–way ANOVA, which has revealed that there are significant differences for wing length and sex comb tooth number in males and for all the traits in females except sternopleural bristle number (Table 4). The magnitude of positional fluctuating symmetry (PFA) differs

significantly among the populations for sternopleural bristle number (males $F_{6, 343} = 2.30$, $P = 0.034$, females $F_{6, 343} = 3.098$, $P = 0.006$) and also for sex comb tooth number in males ($F_{6, 343} = 2.632$, $P = 0.017$). We then calculated the composite fluctuating asymmetry for all the traits in males and females separately for each population. To determine whether there is any difference among the populations for multiple traits one way ANOVA was employed, which revealed that there is significant difference among the populations for males and females (Table 5). There are significant differences between males and females for CFA values. To test this difference statistically, we have performed t-test between the two sexes, which revealed that there are significant differences between males and females ($t = -1.996$, df = 2798, $P = 0.046$).

Table 3. Mean signed FA (R-L), skewness and kurtosis in morphological traits studied in *D.ananassae*

Traits	Sex	Mean ± SE	P¶	Skewness	Kurtosis
SBN	M	-0.111± 0.062	0.072	0.074	0.590
	F	0.134 ± 0.065	0.0645	-0.027	0.724
WL	M	-0.008 ± 0.07	0.914	-0.445	8.90
	F	0.054 ± 0.08	0.492	0.067	4.98
W/T	M	0.0036 ± 0.003	0.304	-14.206	241.38
	F	0.0009 ± 0.001	0.505	0.081	4.493
ST	M	1.349± 0.232	0.061	0.094	-0.203
	F	-0.17 ± 0.119	0.151	0.0063	1.345
PFA_B	M	0.004 ± 0.003	0.182	16.11	286.82
	F	0.012 ± 0.009	0.218	0.149	0.318
PFA_{SCTN}	M	0.0343 ± 0.019	0.69	0.362	1.112

P¶ Evaluating H_0 : mean (R-L)=0; SBN: Sternopleural bristle number; WL: Wing length; W/T: Ratio of wing length and thorax length ; ON: Ovariole number; SCTN: Sex comb tooth number; PFA_B : Positional fluctuating asymmetry for SBN; PFA_{SCTN}: Positional fluctuating asymmetry for SCTN; M : Males; F: Females.

Discussion

The aim of the present study was to answer the following questions: 1) Does inbreeding have any effect on the trait size of morphological traits? 2) Does inbreeding affect the fluctuating asymmetry? If so, then, 3) whether the levels of fluctuating asymmetry increase in the populations that have spent more generations in the lab than those which are of recent origin? and finally, 4) whether the effect of inbreeding is sex and trait specific?

It is evident from the Table 2, that there are significant differences among the laboratory populations for sternopleural bristle number (except males), wing length, wing to thorax ratio, sex comb tooth number, and ovariole number suggesting that there is genetic heterogeneity among the laboratory populations. However, we have not found any effect of the inbreeding on the size of the morphological traits in either males or in females. However, inbreeding is well known to affect the variance among individuals (Whitlock and Fowler, 1999). The inbred individuals show greater variability than outbred in most of the characters (Lerner, 1954). However, there is no increase in variance or coefficient of variation in the morphological traits in males and females (analysis not shown).

Table 4 . Analysis of Variance for the differences in relative FA among the populations in *D.ananassae.*

Trait	Source	SS	df	MS	F
Males					
SBN	Between strains	0.0953	6	0.0159	1.738
	Within strains	3.136	343	0.009	
	Total	3.232	349		
WL	Between strains	0.017	6	0.0028	8.833*
	Within strains	0.111	343	0.00032	
	Total	0.128	349		
W/T	Between strains	0.061	6	0.0102	
	Within strains	2.859	343	0.0083	1.219
	Total	2.92	349		
SCTN	Between strains	0.554	6	0.0923	20.20*
	Within strains	1.567	343	0.00457	
	Total	2.121	349		
Females					
SBN	Between strains	0.082	6	0.0137	1.430
	Within strains	3.274	343	0.0095	
	Total	3.356	349		
WL	Between strains	0.0168	6	0.0028	14.54*
	Within strains	0.0659	343	0.0002	
	Total	0.083	349		
W/T	Between strains	0.102	6	0.017	10.83*
	Within strains	0.612	343	0.002	
	Total	0.719	349		
ON	Between strains	0.439	6	0.073	4.877*
	Within strains	5.145	343	0.015	
	Total	5.584	349		

*P<0.001, Significant after Bonferroni correction P<0.05.

Further, we have measured the levels of developmental instability by calculating the fluctuating asymmetry in different morphological traits in laboratory populations, which revealed that there are significant differences among the laboratory populations for wing length and sex comb tooth number in males and in females for all the traits except sternopleural bristle number (Table 4) implying that FA can be highly trait specific in *Drosophila* (Woods *et al.*, 1999; Vishalakshi and Singh, 2006, 2008 a, b, c, d). There are significant variations in positional fluctuating asymmetry, PFA for sternopleural bristle number in both males and females, and sex comb tooth number in males. The positional fluctuating asymmetry reflects the fidelity of buffering mechanisms operating at the interface of interconnected pathways; thus, it may reflect the genotype-specific ability to integrate such pathways (Polak and Starmer, 2001).

Table 5. Analysis of Variance on a composite FA index created by summing FA values across the four traits in both males and females of *D. ananassae.*

	SS	df	MS	F
Males				
Between strains	0.191	6	0.0319	4.124**
Within strains	10.76	1393	0.0072	
Total	10.956	1399		
Females				
Between strains	0.158	6	0.0263	2.818*
Within strains	12.977	1393	0.0093	
Total	13.134	1399		

P<0.01; ***P* <0.001

Interestingly, the fluctuating asymmetry values are more in sex comb tooth number in males and ovariole number in females in comparison to the non-sexual traits (sternopleural bristle number, wing length, and W/T ratio), supporting our previous findings in *D. ananassae* (Vishalakshi and Singh, 2006, 2008 a, b, c, d). This can be explained as different traits have different developmental windows, in which the developmental stability of a trait is more vulnerable to stress as their development is controlled by distinct gene complexes (Parsons, 1990). Furthermore, different traits are exposed to different degrees of stabilizing selection and canalization depending on their functional significance. For example, greater functional significance to the organism is subject to stronger selection (Waddington, 1957). In the present study, W/T ratio (related to flight capacity) and WL have less fluctuating asymmetry in comparison to SBN, SCTN, and ON. It is evident from Figure 2 d that for sexual traits females show more fluctuating asymmetry than males. It seems that the effect of inbreeding is more in ovariole number (a fitness trait) than the sex comb tooth number in males, which is a secondary sexual trait. Sex comb tooth number is primarily structures adapted for grasping the female securely during the act of intromission. A row of bristles was found near the tip of the female ovipositor that may act as an anchor to the male sex comb bristle during copulation (Mishra and Singh, 2006). Nevertheless, it is widely known that the degree of fluctuating asymmetry in sex comb tooth number in males reveals the individual quality and, therefore, can be used as a reliable signal by females in mate choice (Møller and Swaddle, 1997). But we have found that the size of the sexual trait is a more reliable indicator of individual quality in sexual selection rather than fluctuating asymmetry in *D. ananassae* (Vishalakshi and Singh, 2008a).

The levels of developmental instability are affected by many factors (Leamy and Klingenberg, 2005), but inbreeding is the major factor under the laboratory conditions. In the present study, the laboratory populations were being maintained by periodic transfer of about 50 flies from the old culture bottles to the fresh ones in each generation. Therefore, it might be argued that these populations have become monomorphic due to loss of unfixed genetic variants. Therefore, the older populations (BH-84, BA-87, and KO-93) should show higher levels of fluctuating asymmetry than those of recent ones (PC-05 and MB-06). However, in sternopleural bristle number (see Figure 2a) the SK (02) population, which is neither older than BH or BA nor more recent than MB and PC, shows the maximum fluctuating asymmetry. In constrast to this, in the traits like wing length and wing to thorax ratio, older populations have higher FA than the recent strains. Moreover, in the sexual traits, in males BH-84 being the oldest population has the lowest FA and the levels of FA are similar in the BA-87 and PC-99. Similarly in females, PC-99 shows higher FA, which is neither

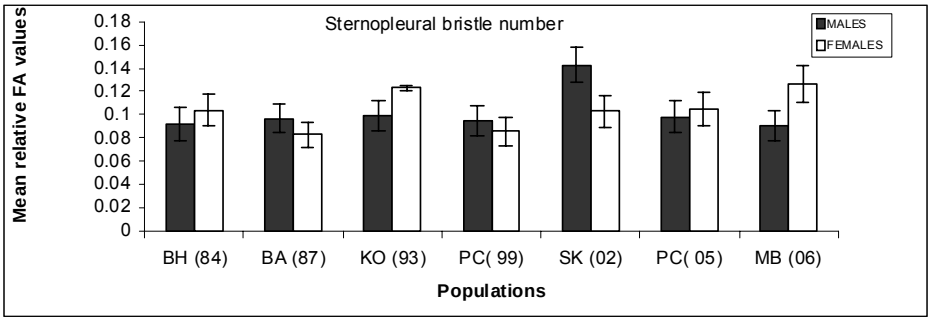
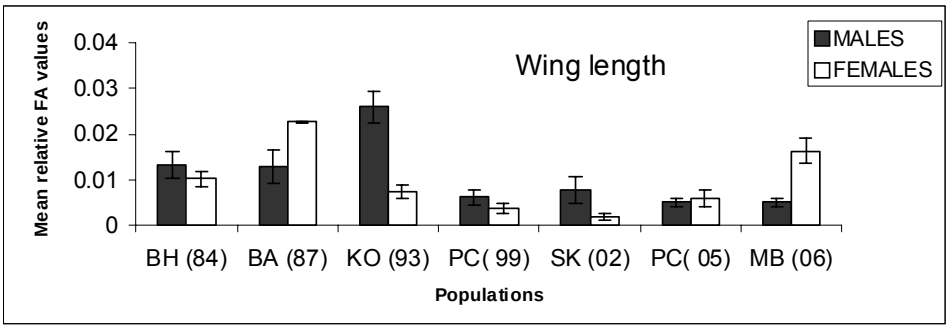


Figure 2 a)



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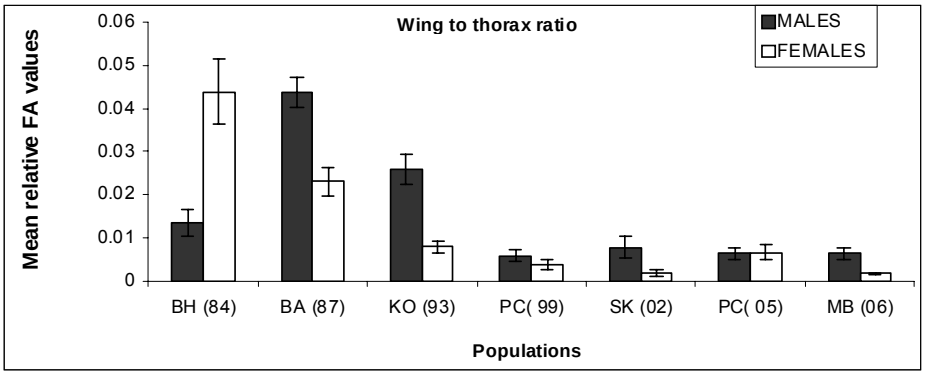

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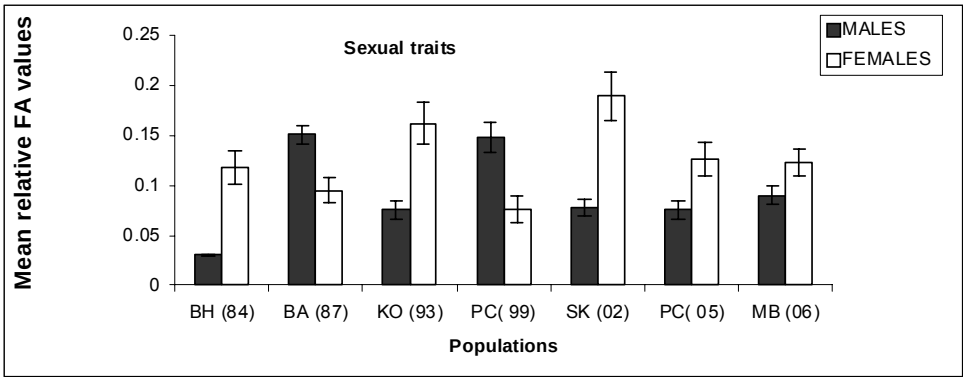

Figure 2 d)

Figure 2. Mean and SE (shown by error bars) of relative fluctuating asymmetry of different populations in a) sternopleural bristle number, b) wing length, c) wing to thorax ratio, and d) sexual traits of *D. ananassae.*

older than BH-84 and BA-87 nor recent than PC-05 and MB-05. Nevertheless, we have not found a consistent increase in the levels of fluctuating asymmetry in the morphological traits in the older populations from the recent ones, but the effect of inbreeding on fluctuating asymmetry seems to be trait and sex specific. Moreover, it has been already reported that inbreeding does not have any effect on fluctuating asymmetry in many organisms, *e.g*., *Drosophila,* sand cricket, and other organisms (Fowler and Whitlock, 1994; Gilligan *et al*., 2000; Leamy *et al*., 2001; Reale and Roff, 2003; Vishalakshi and Singh, 2006).

Thus, the result of the present study added to the growing literature on the relation between fluctuating asymmetry and inbreeding and rejects the proposition that inbreeding should cause a decline in developmental stability which will be reflected by increased fluctuating asymmetry as suggested by Møller (1997). Further, we suggest that in order to measure the real effect of inbreeding, fluctuating asymmetry in morphological traits should be complemented with other indicators such as life history traits.

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