Natural genetic variation in fluctuating asymmetry of wing shape in *Drosophila melanogaster*
Abstract

Fluctuating asymmetry (FA), defined as random deviation from perfect symmetry, has been used to assay the inability of individuals to buffer their developmental processes from environmental perturbations (i.e., developmental instability). In this study, we aimed to characterize the natural genetic variation in FA of wing shape in *Drosophila melanogaster*, collected from across the Japanese archipelago. We quantified wing shapes at whole wing and partial wing component levels and evaluated their mean and FA. We also estimated the heritability of the mean and FA of these traits. We found significant natural genetic variation in all the mean wing traits and in FA of one of the partial wing components. Heritability estimates for mean wing shapes were significant in two and four out of five wing traits in males and females, respectively. On the contrary, heritability estimates for FA were low and not significant. This is a novel study of natural genetic variation in FA of wing shape. Our findings suggest that partial wing components behave as distinct units of selection for FA, and local adaptation of the mechanisms to stabilize developmental processes occur in nature.

Keywords

Animal model ∙ Geometric morphometrics ∙ Heritability ∙ Natural genetic variation ∙ Wing shape
Introduction

Fluctuating asymmetry (FA), defined as random deviations from perfect symmetry, has been observed in many organisms. Because corresponding body parts on the left and right sides of a bilaterally symmetric organism presumably share the same genetic and physical environments, FA is believed to reflect the inability of individuals to buffer their developmental processes from environmental perturbations (i.e., developmental instability) (Whitlock 1996; Palmer and Strobeck 1997; Lens et al. 2002; Fuller and Houle 2003; Klingenberg 2003; Van dongen 2006). The ability to stabilize developmental processes and produce morphological traits with high reproducibility, i.e., smaller FA, is expected to be adaptive under disruptive and fluctuating selection (Pelabon et al. 2010). FA has been reported to relate to a wide range of genetic and environmental stresses (Leary and Allendorf 1989; Lens and Van Dongen 2000), and is a popular tool to estimate fitness of organisms (Clarke 1998; Møller and Thornhill 1998), although some inconsistent FA-fitness relationships have been pointed out (Fowler and Whitlock 1994; Vollestad et al. 1999; Bjorksten et al. 2000). The genetic basis of FA has been studied in various organisms, such as plants, insects, and mammals (Møller and Thornhill 1997; Leamy et al. 1998; Leamy and Klingenberg 2005); Drosophila wings are the most intensively studied model system (Debat et al. 2009).

Although no significant additive genetic variation was estimated for FA of wing shape in D. melanogaster (Woods et al. 1998), Carter et al. (2009) observed a significant increase in FA of
wing shape by inbreeding, suggesting the existence of genetic factors controlling FA. HSP90, a molecular chaperone, was suggested to buffer developmental fluctuations in morphological traits in diverse species such as *Drosophila*, *Arabidopsis* and zebrafish (Rutherford and Lindquist 1998; Queitsch et al. 2002; Yeyati et al. 2007). However, in most experimental settings, the reduction of HSP90 activity did not affect FA of wing shape in *D. melanogaster*. Debat et al. (2006) concluded that Hsp90 is not the major regulator of FA. Takahashi et al. (2010) recently identified another heat shock protein gene *Hsp67Ba* as having a significant effect on FA of wing shape. In addition, some genomic regions of *D. melanogaster* showed potential to affect FA of wing shape (Breuker et al. 2006; Takahashi et al. 2011; Takahashi et al. in press). The genetic basis for FA has been so far investigated using mutant analysis or RNAi approach targeting candidate genes, while little is known about natural genetic variation in FA of wing shape.

Natural genetic variation in FA of wing shape has been investigated in a few studies but is not confirmed (Woods et al. 1998; Debat et al. 2008). A possible reason for this is that the range of sample collection was too limited to cover regions with different degrees of environmental stresses where local adaptations of developmental stability have occurred. Wings of *Drosophila* can be subdivided into several compartments that are subjected to different genetic controls and behave as distinct units of selection (Cavicchi et al. 1985; Cavicchi et al. 1991; Garcia-Bellido et al. 1994; Guerra et al. 1997; Pezzoli et al. 1997). These compartments may have independent molecular
mechanisms for stabilizing developmental processes. Comparison of wild *D. melanogaster* strains from widespread geographical locations and measurement of relevant morphological traits of wings may have a greater potential to uncover natural genetic variations in FA of wing shape.

In this study, we aimed to characterize the natural genetic variation in FA of wing shape in *D. melanogaster*. We used 20 wild strains of *D. melanogaster* collected from across the Japanese archipelago (latitudes from 24°N to 43°N). We quantified wing shape traits at whole wing and partial wing component levels, the stability of which may be regulated by different mechanisms, and evaluated mean and FA of these traits for each strain. We also estimated the heritability of the mean and FA of these traits for the wild strains. Significant genetic variation was found in all the mean wing traits in both males and females. We found significant natural genetic variation only in FA of "crossvein position," the relative position of the posterior crossvein. Heritability estimates for mean shapes were significant in two and four out of five indices in males and females, respectively. On the contrary, heritability estimates for FA were extremely low and not significant. Our findings suggest that partial wing components behave as distinct units of selection for FA, and local adaptation of the mechanisms to stabilize developmental processes occur in nature.

Materials and Methods
The flies used in this study were derived from 20 wild strains of *D. melanogaster* collected from across the Japanese archipelago (latitudes from 24°N to 43°N; Table 1) and maintained in EHIME-Fly, the laboratory for *Drosophila* resources at Ehime University. All the wild strains used in this study were established as iso-females lines, and the generations maintained before our experiments ranged from about 60 to 260, indicating that they were highly inbred strains (M. Watada, personal communication). After we obtained the strains, they were kept under constant light at 23°C in incubators in plastic vials (95 mm height, 24 mm diameter) containing 10 ml of fly medium comprising dried yeast, soy flour, cornmeal, agar, malt extract, and dextrose.

Among-strain genetic variation in wing shape

Experimental conditions

To evaluate natural genetic variation in wing shape, we measured wing shape of each wild strain and calculated among-strain variation. Because larval density is known to affect wing shape in *D. melanogaster* (Bitner-Mathe and Klaczko 1999), we introduced 100 eggs into each vial to control the density effect under constant light at 23°C with the same food medium described above. We set up three replicate vials for each strain and collected emerging adults 10 days after eclosion, and took photographs of wings as described below.
To quantify wing shape using a landmark-based morphometric approach, we captured wing images and obtained landmark coordinates. First, we anesthetized the flies and immobilized their one wing between a slide glass and a cover slip using a simple suction device, wing grabber (Houle et al. 2003). The wing images were then captured with a digital camera, DP25 (Olympus Corporation, Tokyo, Japan), attached to a microscope, SZ61TR (Olympus Corporation, Tokyo, Japan). Right wing images were horizontally flipped to align the orientation of the right and left wing images. We captured wing images of 15 individuals from each strain and each sex. The $x$ and $y$ coordinates for 18 landmarks on a wing (Fig. 1a) were obtained with an automated image-analysis system, Wingmachine (Houle et al. 2003). In this system, a priori B-spline model was fitted to each of the wing images using the pixel brightness of the reversed and filtered images (Lu and Milios 1994; Houle et al. 2003). For the B-spline fitting, Wingmachine requires the $x$ and $y$ coordinates of the basal two landmarks (landmark 9 and 14 in Fig. 1a). Because the acquisition of those landmarks needs to be done manually, this process can be a major source of a measurement error. To evaluate the measurement error, we repeated this landmark acquisition procedure twice. A Procrustes ANOVA (Klingenberg and McIntyre 1998) was performed to assess the relative amount of directional asymmetry (DA), FA and measurement error in wing shape variation. In this analysis, we used individuals, sides, and their interaction term, and measurement error as independent variables,
and added sums of squares across all the landmarks coordinates, assuming equal and isotropic
variation at each landmark. In the current study, the B-spline model fitting on an image was
conducted twice and the average coordinates were used in subsequent analyses to minimize the
measurement error.

Shape analysis

Because the development of partial wing components of *D. melanogaster* are regulated by partially
independent molecular mechanisms (Trotta et al. 2005), the degree of natural genetic variation differ
among the partial components of a wing. To evaluate natural genetic variation at whole wing and
partial wing component levels separately, we quantified wing shape with all the landmarks and
subsets of the landmarks.

Whole wing analysis

In the wing shape analysis based on all the landmarks, we performed the Procrustes generalized least
squares procedure to eliminate the effect of translation, scaling, and rotation from the landmark
configurations, and to extract the non-allometric effect of the shape change in the dataset. In short,
the procedure can be described as follows (Klingenberg and McIntyre 1998). First, all the landmark
configurations were scaled to a unit size. Then, the centroids (or center of gravity) of the
configurations were superimposed. The configurations were then rotated to minimize the sum of
squared deviations of the landmarks of each of the configurations from the homologous landmarks
of the overall consensus (mean) configuration. The resulting Procrustes coordinates were used for
the whole wing shape analysis.

To evaluate the among-strain variation of each landmark, we performed principal
component analysis (PCA). This analysis extracts features of shape variation as a set of new shape
variables, the principal components (PCs), which are uncorrelated to one another and successively
account for maximal amounts of variation. Because a small subset of PCs may be sufficient to make
up most of the total variation, PCA is an effective method for data reduction, which is particularly
important for shape analysis because of the large number of variables (twice the number of
landmarks for 2D data; 36 variables in this study). The landmarks with strong correlation with
dominant PCs would be candidates of representative landmarks in wing shape variation. For the
purpose of visualization of the shape variation, we used the first and second PCs. Since the first PC
(PC1) explained most of the variation in the original landmark configurations (20.4% and 25.5% of
the variation in males and females, respectively), we used the PC1 score as a whole wing shape
index.

Partial wing component analysis

To quantify partial wing shape components, we used four wing shape indices using subsets of the
landmarks. The first index, "elongation index" (Debat et al. 2008), represents the ratio of wing
length to width (Fig. 1b) and was computed as follows,
\[ I_1 = \frac{d[2,13]}{d[1,14]} \]

where, \( d[a, b] \) is the linear distance between landmarks \( a \) and \( b \). This trait has often been used in previous studies (Debat et al. 2008) because of its relative ease of measurement. The second index, "crossvein position" that represents the relative position of the posterior crossvein (Fig. 1c; Pelabon et al. 2006) was computed as follows,

\[ I_2 = \frac{d[11,6]/d[11,3]+d[10,5]/d[10,4]}{2} \]

The third and fourth indices represent the proportion of wing compartments relative to the whole wing area. The third trait, "anterior compartment size," represents the proportion of anterior compartment area (surrounded by the landmarks 1, 2, and 16; Fig. 1d) relative to the whole wing area (surrounded by the landmarks 1, 2, 3, 4, 9, and 14; Fig. 1e). The fourth trait, "posterior compartment size," represents the proportion of posterior compartment area (surrounded by the landmarks 3, 4, 5, and 6; Fig. 1d) relative to the whole wing area. The area surrounded by landmarks 1, 2, 3, \ldots n was calculated:

\[ S = \frac{[(x_n-x_2)y_1+\sum_{k=2}^{n-1}(x_{k-1}-x_{k+1})y_k+(x_{n-1}-x_1)y_n]}{2} \]

where, \( x_k \) and \( y_k \) are the \( x \) and \( y \) coordinates of landmark \( k \). Third and fourth indices were expressed as follows:

\[ I_3 = \frac{S_{\text{anterior}}}{S_{\text{total}}} \text{ and } I_4 = \frac{S_{\text{posterior}}}{S_{\text{total}}} \]

Fluctuating asymmetry
Prior to the calculation of FA, we checked for the presence of DA, directional deviations from bilateral symmetry (Klingenberg and Zaklan 2000), and antisymmetry (AS), the two sides are always different but without a predictable direction to the differences. We performed Kolmogorov-Smirnov tests to examine whether the distribution of the signed asymmetry (difference between index values on the left and right wings) of each index deviated from normal distribution with mean zero. As a result, we observed no significant deviation from normal distribution with mean zero, indicating that the signed asymmetry could be treated as FA rather than a mixture of FA, DA and AS. In both whole wing and partial wing component analyses, FA was evaluated as absolute difference between index values on the left and right wings.

**Analysis of among-strain variation**

Diversification in the mean and FA of the wing shapes among strains was investigated using one-way ANOVA with strain as a random effect. Although the strains were from wide latitudinal range across Japanese archipelago, latitude was not considered as the source of variation. This is because latitudinal cline in mean and FA of the wing shapes was not detected by regression analyses (correlation coefficients ranged from -0.2 to 0.16 and not significant in all the cases). In the present analysis, the following model was used:

\[ w_{ij} = \mu + \alpha_i + \epsilon_{ij}, \]

where \( w_{ij} \) is the response variable (whole wing shape, elongation index, crossvein position, anterior
compartment size or posterior compartment size) of the jth replicate observations (individual) from
the ith strain, $\mu$ is the overall mean, $\alpha_i$ is an effect of the ith strain, and $\epsilon_{ij}$ is an unexplained error
associated with the jth replicate observation from the ith strain. A total of 10 analyses were
performed, two sexes and five indices, for mean trait or FA. To retain an experimentwise error rate
of $\alpha = 0.05$, a significance level for each test was determined by setting the comparison-wise error
rate at $\alpha' = 0.005$, based on the Bonferroni procedure.

Correlation analysis among wing traits

If the major source of shape variation at the whole wing level comes from a partial wing component,
a significant correlation between the whole wing and a partial wing trait may be detected. In addition,
shared regulatory mechanisms between partial wing components may cause correlation of their
variation. To examine these possibilities, pairwise correlations among five indices (whole wing
shape index and four partial wing component indices) were tested by using randomization procedure.
For each of the trait pairs, we randomized one of the trait vectors, and calculated a correlation
coefficient. We repeated the procedure for 1000 times and generated the null distribution of the
correlation coefficients. The observed correlation coefficient was judged as significant at $p=\alpha/500$
if it was smaller or larger than the bottom or top $\alpha\%$ of the null distribution. A total of 20 analyses
were performed, two sexes and 10 combinations of indices, for mean and FA of wing traits. To
retain an experimentwise error rate of $\alpha = 0.05$, a significance level for each test was determined by
setting the comparison-wise error rate at $\alpha' = 0.0025$, based on the Bonferroni procedure.

Heritability experiment

Experimental conditions

To estimate the heritability of the wing traits, we used mass bred populations initiated from 20 wild strains *D. melanogaster* as described above. Two males and two females from each strain was used to set up each mass bred population (initiated with 40 males and 40 females). Each mass bred population was maintained in four 250 ml plastic bottles (with 50 ml of the food medium) containing 100-300 individuals to ensure total population size was more than 1000 individuals per generation to maintain the original genetic variation. These populations were maintained for seven generations prior to the heritability experiment under constant light at 23°C in incubators.

Three generations were assayed to obtain heritability estimates for wing traits. Experimental flies for the parental generation were reared at a standard density (100 eggs per vial), and emerging flies were anesthetized with CO$_2$ and collected as virgins. Wing shapes of 36 male flies and 36 females were measured and then used to establish 36 pair matings (families). Each pair was placed into a vial containing 10 ml of the food medium and allowed to lay eggs under constant light at 23°C. The density of the eggs was checked to prevent overcrowding and the parental flies were removed from the vials 24 hours after introduction. Parental pairs were allowed to lay eggs for
an extra 12 hours when egg density was too low. Emerging adults from each vial were collected as
virgins, and their wings were measured before the mating for the third generation. The mating pairs
were chosen from different families to avoid sib matings. Finally, the emerging grand-offspring
generation was collected and their wings were measured.

Estimation of heritability with animal model

Additive genetic variance for wing shape was estimated using a three-generation design. The animal
model method (Kruuk 2004) was adopted to estimate narrow-sense heritability ($h^2$) using all known
kin relationships among individuals. The animal model can divide the phenotypic variance into
additive genetic, environmental, and other fixed and random variances. We used a univariate animal
model of the form:

$$ y = Xa + e, $$

where, $y$ is a vector of phenotypic values on all individuals, $a$ is a vector of the additive genetic
effect, $e$ is a vector of residual errors, and $X$ is the corresponding design matrix (of 0s and 1s) that
relates the appropriate effects to $y$. The model was run under the Wombat program (ver. 1.0; Meyer
2007).

Heritability estimation for the mean and FA of the whole and partial wing components was
performed separately for males and females. Narrow-sense heritability was estimated as $h^2 = VA /$
$(VA + VE)$ (Houle 1992). In the analysis, significance of the heritability estimate was tested by using
the mean and the standard error. To retain an experimentwise error rate of $\alpha = 0.05$, a significance

evel for each test was determined by setting a comparison-wise error rate at $\alpha' = 0.01$ based on the

Bonferroni procedure. The heritability estimate was considered significant if the approximate 99%

confidence interval (Wilson et al. 2010), the mean +/- 2.58 SE, does not include zero.

**Genetic correlation**

To evaluate whether different wing traits share common morphogenic mechanisms, we

estimated genetic correlations between traits. The genetic correlation was estimated based on the

cross-variance obtained from the product of the trait A score in parents and the trait B score in

offsprings, and the covariances of offspring and parents for each of the characters (Falconer and

Mackay 1996). In the current study, the cross-variance was calculated as the arithmetic mean of the

reciprocal cross-variances between traits. To test the significance of the observed genetic correlation,

we performed randomization test. We randomized one of the trait vectors, and calculated a genetic

correlation using the randomized dataset. We repeated the procedure for 1000 times and generated

the null distribution of the genetic correlation for each trait-pair. The observed genetic correlation

was judged as significant at $p = \alpha / 500$ if it was smaller or larger than the bottom or top $\alpha$% of the

null distribution. A total of 20 analyses were performed, two sexes and 10 combinations of indices,

for mean and FA of wing traits. To retain an experimentwise error rate of $\alpha = 0.05$, a significance

level for each test was determined by setting the comparison-wise error rate at $\alpha' = 0.0025$, based on
Results

Measurement error

All the main factors in the Procrustes ANOVA were statistically significant (Table 2). This result indicates that there were significant DA and FA in our dataset although significant DA was not detected in the wing traits calculated based on the dataset as described above. The contribution of measurement error to the overall shape variation was small in both sexes.

Patterns of variation in landmarks

PCA extracted features of wing shape variation, indicating that most variation was concentrated in a few dimensions. In both males and females, the first five PCs accounted for 70% of the total variance. Fig. 2 displays the features of variation associated with the first and second PCs, as plots of the PC coefficients superimposed onto a drawing of the wing. PC1 was primarily affected by the large variability of anterior crossvein position, associated with the movement of landmarks 7 and 8, moved along the proximo-distal axis and also associated with the variation of landmark 1 in males (Fig 2a). PC2 was primarily affected by the variability of the posterior crossvein position, associated
with the movement of landmarks 5 and 6, moved along the proximo-distal axis and also associated with the movement of landmark 1 in males (Fig 2b). In females, PC1 was primarily affected by the variability of anterior crossvein position (landmarks 7 and 8), moved along the proximo-distal axis and also associated with the variation of landmarks 1 and 3 (Fig 2c). PC2 was primarily affected by the proximo-distal movement of posterior crossvein position (landmarks 5 and 6) and also associated with the proximo-distal movement of anterior crossvein (landmarks 7 and 8; Fig 2d).

Patterns of variation in wing traits

In both males and females, the mean of all the wing shape indices showed highly significant diversification among strains (Table 3). Significant correlations were detected between the whole wing shape and partial wing components: whole wing shape-crossvein position, and whole wing shape-posterior compartment size in both sexes, and whole wing shape- anterior compartment size in only males (Table 4). Several significant correlations were found among partial wing components: crossvein position-anterior compartment size, crossvein position-posterior compartment size, and anterior compartment size-posterior compartment size were all significantly correlated in both sexes, and elongation index-posterior compartment size was significantly correlated in females alone (Table 4).

In both males and females, no significant diversification in FA of the whole wing shape
was detected (Table 5). As for partial wing component FAs, only crossvein position in females showed significant diversification among strains (Table 5). No significant correlation was found between FAs of whole wing shape and partial wing components (Table 6). Several significant correlations were found among FAs of partial wing components: elongation index-posterior compartment size and crossvein position-anterior compartment size were correlated in males, and crossvein position-posterior compartment size and anterior compartment size-posterior compartment size were correlated in females (Table 6).

**Heritability of wing traits**

The heritability estimate for mean whole wing shape was significantly larger than zero in females but not in males (Table 7). For the mean partial wing components, heritability estimates for crossvein position and posterior compartment size in males and crossvein position, anterior compartment size and posterior compartment size in females were significantly larger than zero (Table 7). The significant heritability estimates for mean traits ranged from 0.426 to 0.827 depending on the trait and the sex (Table 7). The estimates of genetic correlation among the mean wing traits were not significant in all the cases. In contrast, the heritability estimates for FA of the wing shape traits were small, and not significantly different from zero for all indices in both males and females (Table 8). The estimates of genetic correlation among the FA of wing traits were not
Discussion

In this study, we investigated whether there was natural genetic variation in FA of wing shape in *D. melanogaster*. All the means of wing traits showed highly significant diversification among wild strains in both males and females, indicating large natural genetic variation in these traits. Although the measures of wing morphology were somewhat different, previous studies also observed similar natural genetic variation in the wing traits (Pezzoli et al. 1997; Woods et al. 1998; Debat et al. 2008). A recent expression study reported that 164 of 1,335 genes changed their expression significantly during wing morphogenesis and differentiation (Butler et al. 2003), suggesting that a large number of genes are potentially involved in wing morphogenesis, and could be a source of natural genetic variation. In contrast, significant diversification in FA among wild strains for FA was only detected for crossvein position in females. This result, which is consistent with previous results (Woods et al. 1998; Debat et al. 2008), suggests that natural genetic variation in FA was limited to a partial wing component, and could not be detected only by assessing the whole wing shape FA.

In the correlation analyses for mean traits, we found a couple of significant correlations between the means of whole wing shape and partial wing components and also among partial wing
components. On the other hand, we found no significant correlation between FAs of the whole wing shape and partial wing components, and a smaller number of significant correlations among partial wing components than for mean traits. In contrast to the result from among-strain genetic variation experiments, we could not find significant genetic correlation among the means and FAs of these traits in the heritability experiment. These results indicate that some of the partial wing components of *Drosophila* are subjected to at least partially different genetic control (Garcia-Bellido et al. 1994; Guerra et al. 1997; Pezzoli et al. 1997). The results also suggest that the genetic regulation of FA was more independent among partial wing components than of mean traits, resulting in no significant correlation between the FAs of whole wing shape and partial wing components. Although we found several significant correlations between partial wing components, the correlations between crossvein position and posterior compartment size in mean and FA may be an artifact due to shared landmarks on the posterior crossvein (landmarks 5 and 6). The significant correlation between the anterior and posterior compartment sizes, found both in mean and FA in females, suggests that they may share not only morphogenic but also developmental buffering mechanisms. The significant correlation between elongation index and posterior compartment size, found for FA in males, but not for mean traits, suggests that morphogenic and stabilizing factors were independent in this case. These results emphasize that some partial wing components of *Drosophila* wings are distinct units of natural selection, subjected to different genetic control. So far,
no gene has been found to affect wing shape FA in a wing compartment-specific manner. Genes that show restricted expression patterns in multiple wing compartments are potential candidates for such an effect. Future investigation of such genes may elucidate how *Drosophila* wings respond to natural selection of developmental stability in nature.

In the current study, results of the heritability estimates did not always support the results of the among-strain diversification (e.g., significant among-strain diversification detected in mean whole wing shape, elongation index, and anterior compartment size in males, and elongation index in females, but no significant heritability estimates for them). As for FA of the crossvein position, we found significant among-strain diversification, but no significant heritability. These discrepancies might come from the three-generation approach of the heritability estimation, which allows recombination between homologous chromosomes from different strains. If multiple genes contributed to the genetic diversification in these traits, recombination during the experimental crosses might disrupt a set of coadapted alleles, and reduce the additive effect of these alleles below the limit of detection. Based on a simulation model, Fuller and Houle (2002) suggest that artificial selection for increased FA is the most powerful approach for the detection of genetic variation in developmental instability. In the future, performing artificial selection on the partial wing components may be necessary to estimate genetic variation in FA at higher resolution.
first time. Our finding that only one component of the wing showed significant genetic variation in
FA suggests that partial wing components behave as distinct units of selection for FA in nature.
Further investigation on how FA of the wing trait is regulated, and subjected to natural selection may
facilitate understanding of the evolution of developmental stability.

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Figure legends

Fig. 1 Landmark positions and wing indices, a Eighteen landmarks on the wing vein junctions, the wing margin, and on the free ends of wing veins, b elongation index, c crossvein position, d anterior and posterior compartment, and e whole wing area

Fig. 2 Principal component analysis (PCA) of variation in landmark positions for individual variability. The diagrams visualize the PC coefficients of each landmark in x and y directions by a line originating at the average location of the landmark (circles). a PC1 in males, b PC2 in males, c PC1 in females, d PC2 in females