Disentangling directional and fluctuating asymmetry in a genome-wide association study

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Abstract

Although directional and fluctuating asymmetry have been frequently assessed independently, they are indeed associated concepts both in theory and in practice. However, they can be difficult to disentangle in genome-wide association studies, where the appropriate shape statistics are not fully developed. Although the usage of Procrustes distances to overcome this problem may be tempting, this does not reliably help to identify the underlying genetic components of directional and fluctuating asymmetries. Here, similarities and differences among different approaches have revealed that the genetic component of the skull asymmetry in this population of mice is mostly associated to fluctuating asymmetry. This is coherent with the previous literature and it adds a note of caution in the study of asymmetry in a genomic context. The results also pointed out at the need of developing a multivariate framework to conduct shape analyses in general and asymmetry tests specifically. The combination of high-dimensional shape data and the vast number of genomic markers makes it challenging but the different statistical errors can hide important biological information.

Keywords: *GWAS, MICE, MULTIVARIATE, GENETICS, SKULL*

1. Introduction

Shape asymmetry is a widely studied aspect of shape in evolutionary studies (Klingenberg, 2015). Different types of asymmetry have been described, being the most common directional and fluctuating asymmetry (from hereon, DA and FA) (Figure 1). FA is by far the most used type of asymmetry since long time ago (Van Valen, 1962). It has been traditionally considered an isolated product of development, naturally stochastic. Since both sides of a structure share the same genes and the same environmental conditions (especially in motile organisms), differences between them may be due to stochastic perturbations during development (Van Dongen, 2006). Therefore, it has been a very attractive phenotypic character to be used in developmental studies, especially in combination with environmental set-ups, under the hypothesis that environmental stressors would increase FA. In that context, it has been used as a proxy for the inability of an organism to buffer its development against that environmental stress. This inability has obvious consequences on the fitness and therefore evolutionary consequences. However, results under this framework have not always yielded a consensus (De Coster *et al.*, 2013), maybe because genetic variation also affects developmental stability but it has not always been taken into account (Polly *et al.*, 2011).

FA can be the only type of asymmetry in a sample if all the variation in asymmetry is random (therefore distributed around the perfect symmetry). However, it can also be centred on a systematic difference between two sides of a structure in a population. This is DA. As an example, we can think about the size of the lungs in humans: the right lung is consistently bigger than the left one. In different words, in humans the size of the right lung is on average bigger than the left lung. There is DA when the mean asymmetry in a population is different from the perfect symmetry. Why a consistent asymmetry (DA) exists at all in a population? Evidences about the evolutionary origin of DA are not conclusive and show that the evolution of DA may depend on the structure and the organism. There have been studies showing that this asymmetry facilitates a correct development

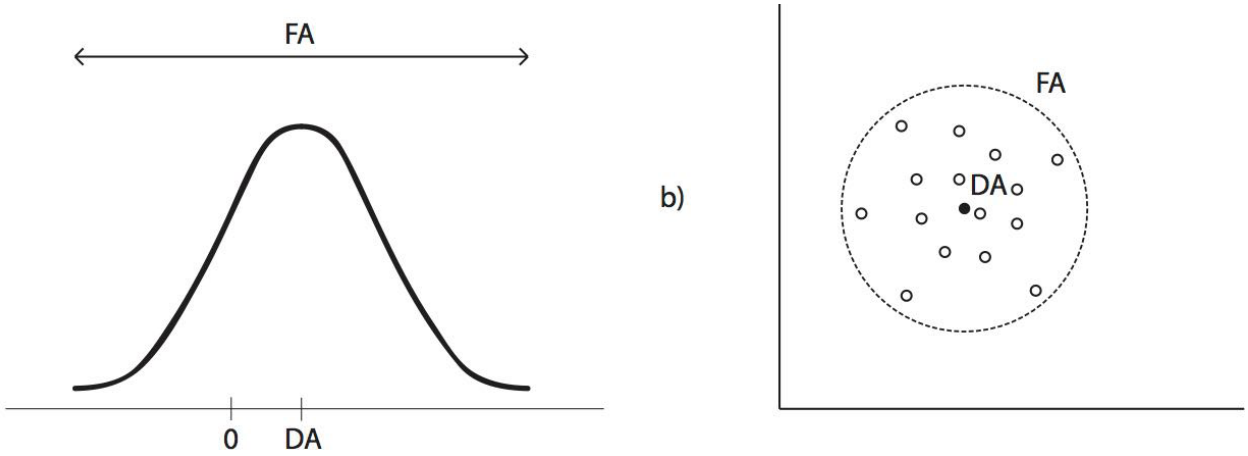

FIGURE **1.** Statistical concepts of directional and fluctuating asymmetry in univariate (a) and bivariate (b) spaces. In (a) we assume that a population has a symmetric component univariate and normally distributed. It presents directional asymmetry (its mean asymmetry is different from 0) and certain fluctuating asymmetry (variation around the mean). In (b) a population is represented by its individuals (dots) in a bivariate asymmetric space. Here again, its mean (DA) and variation (FA, dashed line) are represented.

of a functional organism and therefore it influences its fitness (Gamse *et al.*, 2005). However, non-adaptive hypotheses where DA is a product of developmental trade-offs can be also found elsewhere (Pélabon & Hansen, 2008). The molecular origins of DA, which may be the substrate for natural selection to act on, have been linked to the differential expression of certain genes at both sides of the bilateral organisms (Levin, 2005).

The existence of a genetic component in asymmetry has been proposed since the first studies on it, especially for FA (Grüneberg, 1953, Mather, 1953, Thoday, 1958). This has been shown in first place with the associations between major genetic syndromes and FA (Bock & Bowman, 2006, Miller *et al.*, 2014, Richtsmeier *et al.*, 2005). However, although the study of shape asymmetry based on morphometric methods is a very developed area relatively few studies have been carried on its genetic component (Leamy *et al.*, 2000, Leamy *et al.*, 2015, Leamy *et al.*, 2001). Based on results from traditional morphometrics as well as other characters (Graham *et al.*, 2010), the current consensus about the genetic component of FA suggests that epistasis may be the major biological process involved in its generation (Leamy *et al.*, 2005, Leamy *et al.*, 2015, Van Dongen, 2006). There are, however, empirical studies showing that specific genes are involved in extreme FA (Debat *et al.*, 2011). In the case of DA, less information is available. Few studies have identified additive effects associated to it (Leamy *et al.*, 2000, Shapiro *et al.*, 2004) while others found no response to selection in DA (Tuinstra *et al.*, 1990).

2. Genetic DA in combination with unbalanced sample sizes may bias univariate estimations of FA

Genome-wide association studies (GWAS) in model organisms as mice have been around now for some time (Flint & Eskin, 2012) and represents an excellent opportunity to study the genetic component of asymmetry comprehensively. However, they are not exempt from methodological challenges. The estimation of DA, as most shape characters, needs from multivariate analyses. This is an issue in genomic studies because of the high dimensionality of the phenotypic character added to the vast amount of genomic data. Certain analyses, e. g. the extraction of the population structure (Speed & Balding, 2015), can become too demanding. Theoretical advances for the adaptation of common univariate techniques to shape data in the context of GWAS start to develop (Mitteroecker *et al.*, 2016) and few techniques on the estimation of the genetic structure have been developed for general multivariate data (Runcie & Mukherjee, 2013). Nonetheless, its suitability for shape data is still unexplored. Populations of mice where the population structure has been standardized (Nicod *et al.*, 2016) are currently one safe option to apply geometric morphometric methods in GWAS. The case of FA on its own can be simplified, even if some controversy about its characterization can also be found in the literature (Palmer & Strobeck, 1992, Whitlock, 1996). Since FA consists on random variation, the usage of Procrustes distances (Klingenberg & McIntyre, 1998) from the position

of each individual to the population average as an individual measure of FA is a possibility. The collapse of multivariate data on univariate measures brings a loss of information about direction that under the assumption of random variation may be acceptable. Otherwise, Mahalanobis distances can be used (Klingenberg & Monteiro, 2005).

The possibility of reducing multivariate estimates of FA to univariate measures might induce to think that reliable estimates of genetic FA can be obtained independently of DA. However, it has been shown that both DA and FA are necessarily related in theory but also in practice (Stige *et al.*, 2006). The statistical dependence between DA and FA as well as unbalanced sample sizes in different genetic populations produce artefacts on the estimation of asymmetry variation and bias the results. Therefore, the estimation of DA might be essential even if there is interest in just FA. In presence of genomic DA, unbalanced sample sizes cause the whole population average to be different from the genetic subsamples average. That produces a systematic increasing on the Procrustes distances from the population average to the smaller genetic population. Therefore, artefacts on the estimations of DA and/or FA may appear (figure 2). This systematic increasing would be reflected in an analysis of means, showing DA. Also, depending on the distribution within the genetic populations, this would be reflected in an analysis of variance. The smaller population, further, would show higher variance and therefore higher FA. This is an obvious distortion of the estimation of DA and FA by Procrustes distances, which can yield all kind of statistical errors in a wide different range of situations (figure 2). Unfortunately, controlling for unbalanced sample sizes is not feasible in practice, since it requires a minor allele frequency threshold too high.

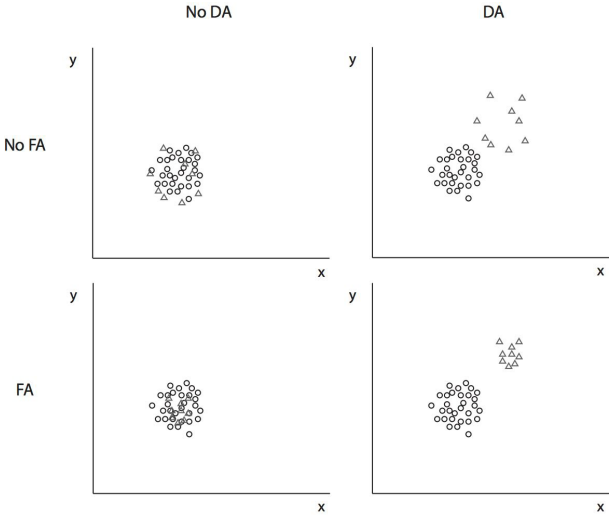

FIGURE **2.** Effect of sampling unbalance on the estimation of DA and FA in two genetic populations (grey triangles and black circles). In a situation of neither DA nor FA (upper left diagram), there are consistent larger distances from the population average to the triangle population (peripherally distributed). Therefore, the usage of Procrustes distances may produce significant results for DA. In a situation of lack of FA but presence of DA (upper right diagram), there is consistently more variation in distances from the population average (~circle population average) to the triangle population. Therefore, Procrustes distances may enhance significant results for FA. In presence of FA (bottom diagrams), biases are analogous but in opposite directions.

3. EMPIRICAL APPLICATION

An approach to prevent the artefacts that the estimation of Procrustes distances can produce in presence of unbalanced sampling and DA is feasible. This consists on the application of two consecutive generalized linear models. First, a multivariate linear model in order to control for the genetic DA is applied. That would remove the combined effect of DA and unbalanced sample sizes, leaving the residuals as a pooled distribution of the variation in the whole population. Then, the estimation of FA for each individual is straightforward: the residual distances (i.e. distances from each individual coordinates to its predictive values) are individual measures of FA free from DA and problems derived from sampling. These univariate values can be used in a second linear model to study the genetic component of FA. This approach has been developed almost twenty years ago (Smyth, 1989) and it has been proposed recently under the name of double-generalized linear model for mapping QTL for the variance of univariate data (Rönnegård & Valdar, 2011). This method also adds some complexity to the reasoning: the linear fitting of the mean and the variance components are done maximizing their likelihood in a loop, so the estimation of the mean part is also dependent on the variance part. This approach seems appropriate when there is an interest on the genetic component of both a character mean and its variability, as might be the case for asymmetry.

In order to test for the practical influence of DA on the estimation of FA and the differences with a double-linear model, these tests are applied to

an empirical dataset. This consists on a population of 692 Carworth Farms White (CFW) mice, where each individual has been genotyped and phenotyped (Pallares *et al.*, 2015a, Pallares *et al.*, 2015b). The convenient particularity of this population is that it has been designed to reduce its population structure as much as possible. This facilitates the usage of multivariate linear models, since the population structure is of no concern (results not shown). The phenotype consists on the shape extracted from 44 landmarks in 3D in the skull. Shape was obtained from a full-generalized Procrustes superimposition with object symmetry (Dryden & Mardia, 1998). Then, the asymmetric component was extracted (Klingenberg *et al.*, 2002). Both the Procrustes superimposition and the asymmetric component collection were run on MorphoJ v1.06 (Klingenberg, 2011). The genomic data consists on the gene dosage for 79 787 SNPs, once the markers with a maximum genotype probability smaller than 0.5 and a minor allele frequency smaller than 2% were removed.

Two different approaches for the estimation of FA are followed. First, a one-step univariate linear model for FA is applied, where individual measures of FA are the Procrustes distances from each individual asymmetric component to the whole population asymmetry average. This would constitute an acceptable estimation of genetic FA considering balanced sample sizes and absence of DA. Then, two consecutive linear models are used. This starts with a multivariate linear model on the whole asymmetric component of each individual in order to remove the effect of genetic DA followed by a univariate linear model for FA using the residual distances. Note that in absence of genetic DA, the results under this approach should be at least similar to the results obtained using the first simple approach. The statistical significance of these results is assessed via permutation test (Churchill & Doerge, 1994). All the results presented as LOD scores. The analyses were performed in R v3.3.1 (R Core Team, 2013).

Our results show a clear correlation between the results from the FA univariate linear model and the residual distances of the double model (figure 3). The correlation between the LOD scores of these two approaches (r = 0.94) is indeed very high. In the other hand, the correlation between the first single univariate model and the multivariate model is extremely low (r = 0.02). It is important to note, however, that even among highly correlated approaches there is still some variation in the results. The 95% genome-wide threshold

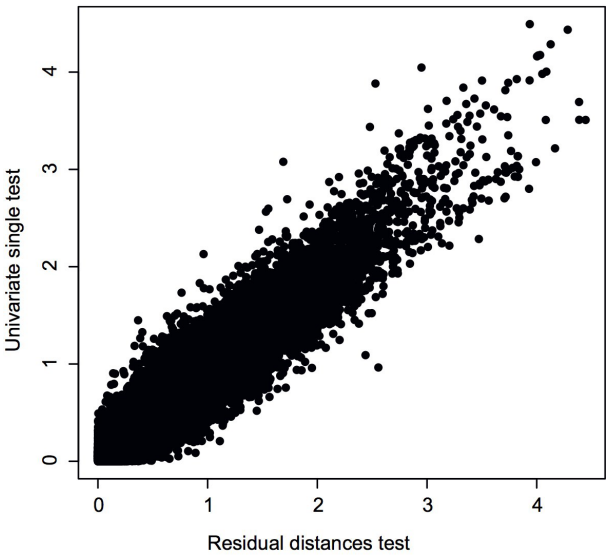

FIGURE 3. The results for the univariate test and the test using residual distances are plotted to see their correlation.

for the single univariate analysis is 6.20 and therefore no marker gives significant results (figure 4a). This is the same for the multivariate model for DA (95% significance threshold = 6.18) and its residual distances (95% significance threshold = 6.24) (figures 4b and 4c).

4. The absence of directional asymmetry in mice skulls might simplify the statistical analyses of FA even in presence of unbalanced samples

The results for the multivariate model show that DA has not an important genetic component in the skull of our mice population. This is coherent some previous findings about mice skull (Mikula *et al.*, 2010, Mikula & Macholán, 2001), although opposite evidence has been found for the mandible (Leamy *et al.*, 2000). In addition, the high correlation between the one-step univariate model and the univariate model estimated from residuals distances suggests that the genetic component of DA is indeed very low. In addition, unbalanced sample sizes have not affected the results obtained from a single univariate model. These are good news, since multivariate analyses are more computing-demanding, especially in the context of genomic studies where thousands of markers are explored.

These results might induce to think that multivariate approaches might not be necessary to estimate FA when DA is subtle. In addition, no

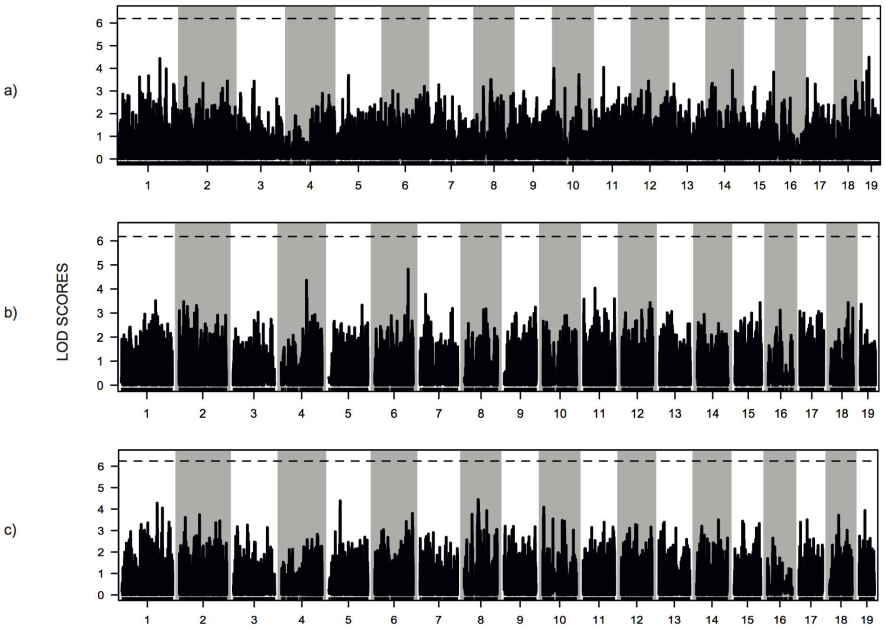

FIGURE 4. Genome scan for the single univariate approach (a), the double linear model with its mean part (b) and its variance part (c). Each line represents the LOD score of one genomic marker. The dashed lines represent the 95% significant threshold for each scan.

special action for controlling sample balance in our empirical dataset has been needed. These are encouraging news since FA has received much more attention than DA since the beginning of the studies about shape asymmetry. It has a more straightforward explanation and it has served as an indicator for environmental stress in other areas as conservation biology (De Coster *et al.*, 2013) or medicine (Klingenberg *et al.*, 2010). The absence of DA would imply that the whole population asymmetry average is equal to the different genetic subpopulation averages. Therefore, all the variation within the population asymmetry would be FA. The collapse of the multivariate characters into Procrustes distances would not distort the FA estimation in favour of one specific genetic population. The small contrast among the results, i. e. the fact that no additive effects seems to be involved in their origin, raises also the question about whether DA and FA share a common genetic architecture and whether that might simplify the analyses. It is also unclear at what extend the genetics of asymmetry is species, structure-dependent or both. Further studies about the genetics of asymmetry would be needed to shed light on how general the situation in this empirical dataset is. Past studies have shown different types of results, both for DA (Shapiro *et al.*, 2004, Tuinstra *et al.*, 1990) and for FA (Debat *et al.*, 2011, Leamy *et al.*, 2015). Despite their popularity in ecological studies and the late advances on the collection of genomic data, the genetics of directional and fluctuating asymmetry is not very well known.

Although the study of this empirical datasets has encouraged the notion that genetic FA can be studied on its own in absence of DA, it is important to note that despite the coherent results among approaches variation among approaches is still present and that may be important. Although the correlations are high, variation in the results can hide some effects that may be of interest especially in nature. There are, in addition, outliers that drastically changed their result in response to different treatment. Therefore, the absence of DA standardization could also disrupt downstream analyses involving asymmetry and enhance artefacts.

6. CONCLUSIONS

The estimation of DA needs to be considered in studies about asymmetry even if there is just interest on FA (Stige *et al.*, 2006). This process is relatively complex because the estimation of position of the population average in the asymmetric space is involved. Therefore, a multivariate approach would be appropriate and its application is currently challenging for shape data in a genomic context. Few techniques are already available to take into account the multivariate nature of the data but some of them are unfeasible for high-dimensional data (Zhou & Stephens, 2014) and others are based on assumptions unexplored for shape data (Runcie & Mukherjee, 2013). Once this technical development will be achieved, the study of the genomics of asymmetry should be able to routinely take into account at the same time the particular nature of shape and genomic data. In order to achieve this, a two-step procedure is proposed

as a reliable way of inferring patterns of DA and extracting the appropriate information to be use in a subsequent FA analysis.

For now, in those populations where genetic DA is absent, univariate methods based on Procrustes distances could be an acceptable used in the estimation of FA without further measures of caution, as it has been the case for the empirical dataset presented here. However, some measures of caution would be necessary to check on the method used to analyse FA, since some variation will still be created by these methods and that in combination with permutation tests can change the significant results obtained. In any case, further studies exploring the genetics of asymmetry will be of great interest to generalize the results presented.

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