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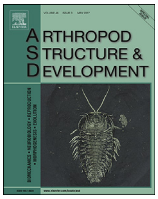

Development and evolution of segmentation assessed by geometric morphometrics: The centipede *Strigamia maritima* as a case study

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ABSTRACT

Using the centipede model species *Strigamia maritima* as a subject of study, we illustrate the potential of geometric morphometrics for investigating the development and evolution of segmentation, with a specific focus on post-embryonic segmental patterning. We show how these techniques can contribute detailed descriptive data for comparative purposes, but also precious information on some features of the developmental system that are considered relevant for the evolvability of a segmented body architecture, such as developmental stability and canalization. Morphometric analyses allow to separately investigate several sources of phenotypic variation along a segmented body axis, like constitutive and random segment heteronomy, both within and among individuals. Specifically, in *S. maritima*, the segmental pattern of ventral sclerite shapes mirrors that of their bilateral fluctuating asymmetry and among-individual variation in associating the most anterior and most posterior segments in diverging from the central ones. Also, among segments, there seems to be a correlation between fluctuating asymmetry and shape variation among individuals, suggesting that canalization and developmental stability are somehow associated. Overall, these associations might stem from a joint influence of the segmental position on the two processes of developmental buffering.

1. Introduction

Segmentation is a key feature of arthropod body architecture, and as such considerable attention has been paid to its development and evolution, also in relation to tagmosis, the morpho-functional regionalization of the main body axis (reviewed in Fusco and Minelli, 2013).

The evolution of segmentation as a body feature, in connection to the developmental process of segmentation itself, is a favourite subject of evolutionary developmental biology (evo-devo), as it is thought that the developmental mechanism of segmentation, along with the network of genetic interactions that controls it, can have significant influence on the evolution of segmentation as a morphological trait (e.g., Davis and Patel, 1999; Peel et al., 2005; Vroomans et al., 2016). However, most of the more recent studies on the development of segmentation have focused on the embryonic (often, early-embryonic) phase of development. This has been done using techniques like in-situ hybridization to study gene expression, parental and embryonic RNA interference to study gene function, and sequencing of transcriptomes and genomes to reveal the complete gene repertoires of some of these animals (Leite and McGregor, 2016). However, despite remarkable recent progresses in developmental biology, especially in comparative developmental genetics, the study of post-embryonic development still remains insufficiently investigated, if compared to the preceding phase of ontogeny. This is particularly unfortunate for arthropods, where developmental processes of segmentation and tagmosis are not restricted to embryogenesis, but continue prominently through post-embryonic life (Minelli and Fusco, 2013).

Comparative analysis of the evolution of segmentation, including segmental patterning, needs other types of data, typically morphometric data, and different analytical approaches, such as those offered by geometric morphometric (GM) methods, a suite of analytical tools that provide a statistical description of biological forms in terms of their size and geometric shape (e.g., Klingenberg, 2010).

Morphological variation along a segmental series (*segment heteronomy*) within an individual has at least two different

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components. The first is *constitutive segmental variation* in size and shape of the elements of the series, corresponding to the more general concept of *target phenotype*, i.e. the phenotype specified by the genetic makeup of the organism and the environmental conditions during its development (Nijhout and Davidowitz, 2003; Fusco and Minelli, 2010). The second is *random segmental variation*, i.e. variation around the target phenotype produced by developmental noise, that can be manifested as deviations from the expected body symmetry, or *fluctuating asymmetry* (see below) (Fusco and Minelli, 2000a; Savriama et al., 2016). Both constitutive and random segmental variation, metaphorically the “signal” and the “noise”, are of high interest for both developmental studies, across ontogenetic stages within species, and evolutionary studies, comparing species in a phylogenetic context.

At the same time, phenotypic variation observed in a population is the outcome of two opposing sets of influences: on the one hand are the sources of variation, including genetic and environmental differences among individuals and developmental noise; on the other hand is a set of developmental processes that buffer those variations, including canalization and developmental stability (e.g., Debat and David, 2001; see below). Because developmental noise is difficult to set apart from environmental effects, a strategy is to focus on within-individual variation among repeated body parts. These parts are indeed genetically identical and are usually facing the same environmental conditions, and thus differ only by stochastic differences attributable to developmental noise (e.g., Van Valen, 1962).

Within-individual deviations from the expected body symmetry are known as fluctuating asymmetry (FA) (e.g., Debat and David, 2001; Debat and Peronnet, 2013; Klingenberg, 2015). In organisms with bilateral symmetry, random deviations from left-right symmetry, or *bilateral FA* (Palmer and Strobeck, 1986), are often employed to investigate *developmental stability*, i.e. the ability of an organism to buffer random perturbations of its developmental process (Nijhout and Davidowitz, 2003; Fusco and Minelli, 2010). However, for segmented animals, or for organisms with a modular body organization in general, other types of body symmetry, specific of their body architecture, can be exploited to study developmental stability by means of FA (Savriama and Klingenberg, 2011). Along their main body axis, arthropods, like other segmented animals (Minelli and Fusco, 2004), present translational symmetry, which can be effectively exploited through the analysis of *translational FA* for the study of developmental stability (e.g., Savriama et al., 2016).

Among-individual phenotypic variation results from the interplay between genetic and environmental influences and canalization. The term *canalization* (Waddington, 1942) refers to the ability of the developmental system to buffer such influences, with the effect of limiting phenotypic variation (e.g., Debat and David, 2001; Fusco and Minelli, 2010). Although the observed variation is thus the manifestation of both the sources of variation and canalization, differences in variation among individuals among groups are often interpreted as differences in canalization (e.g., Clarke, 1998; Debat et al., 2009; Breno et al., 2011; Lazić et al., 2015), assuming that the sources of variation are globally constant.

Whether developmental stability and canalization are independent features of a developmental system has been a contentious issue (e.g., Klingenberg and McIntyre, 1998; Debat et al., 2000; Hallgrímsson et al., 2002). The most commonly used approach to investigate the link between canalization and developmental stability has been to compare, among groups, the levels of variation among individuals and FA. The use of GM methods has provided an additional, more subtle criterion: a similarity in the patterns of shape variation among individuals and between sides within individuals would suggest that similar developmental processes are acting at both levels, and that canalization and stability are related. Conversely, if these patterns are different, then canalization and stability might involve different processes. The literature has so far provided contrasting results (see Debat et al., 2009; Klingenberg, 2015 for reviews). In this context, an obviously appealing feature of segmented animals, is that they offer as many traits as their number of segments to assess this relationship, with the additional question of whether it might differ across segments, and be influenced by the overall heteronomy.

Here, we illustrate the potential of geometric morphometrics for investigating development and evolution of segmental patterning, using the model centipede species *Strigamia maritima* as the subject of study. In particular, we present some general morphometric approaches for the study of size and shape segment differentiation (constitutive heteronomy) and bilateral and translational FA. We focus in particular on the patterns of bilateral shape asymmetry of segments (as a proxy for developmental stability) and shape variation among individuals (as a proxy for canalization), and explore whether they might differ regarding the position of segments along the main body axis.

In the last two decades, together with the pill millipede *Glomeris marginata*, the centipede *S. maritima* (Fig. 1A) has become a favourite subject of myriapod developmental biology, with a series of studies on its embryonic (Chipman et al., 2004; Brena and Akam, 2012) and early-postembryonic (Brena, 2014) development, and developmental gene expression (Brena et al., 2006; Chipman and Akam, 2008; Brena and Akam, 2013; Brena, 2015). *S. maritima* is also the only myriapod with a fully sequenced genome (Chipman et al., 2014). As typical of geophilomorph centipedes (Chilopoda, Geophilomorpha), *S. maritima* presents a highly polymerous and a rather homonomous (i.e., morphologically little-differentiated) segmental body organization, with respect to other arthropods. Nonetheless, as we will show, detailed quantitative morphometric analyses, that can be equally applied to more complex segmental patterns, reveal a surprising richness in segmental patterning, in the relationship between different aspects of developmental control at the level of segments, and in the connections between segmental patterning and developmental control.

The quantitative analysis of arthropod segmental patterning has repeatedly been an object of interest in morphological studies on the group, although always based on traditional (meristic or distance measurements) morphometric analysis (e.g., Enghoff, 1986; Minelli et al., 1996; Berto et al., 1997; Fusco and Minelli, 2000b; Bonato et al., 2011; Ivanenko et al., 2016) and generally considering only one or very few specimens of the same species. Centipede segmental patterning has also been used as a model for developing an index of morphological complexity (Fusco and Minelli, 2000a). On the contrary, the list of studies dedicated to the random deviations from constitutive heteronomy, i.e. studies on FA in relation to segmentation, or translational FA, reduces to a couple of pioneering studies (Astaurov, 1930; Fusco and Minelli, 2000b) and a very recent one (Savriama et al., 2016), the only making use of geometric morphometry.

The selection of GM analyses that we present here applied to *S. maritima* trunk segmentation can all be employed in other segmented organisms, and have thus the potential to generate suitable comparative data for the study of the evolution of segmental patterning and its developmental control in wide phylogenetic contexts.

2. Materials and methods

2.1. Species and sample specimens

The trunk of *S. maritima*, as those of all geophilomorph centipedes, comprises one anterior segment bearing a pair of venomous

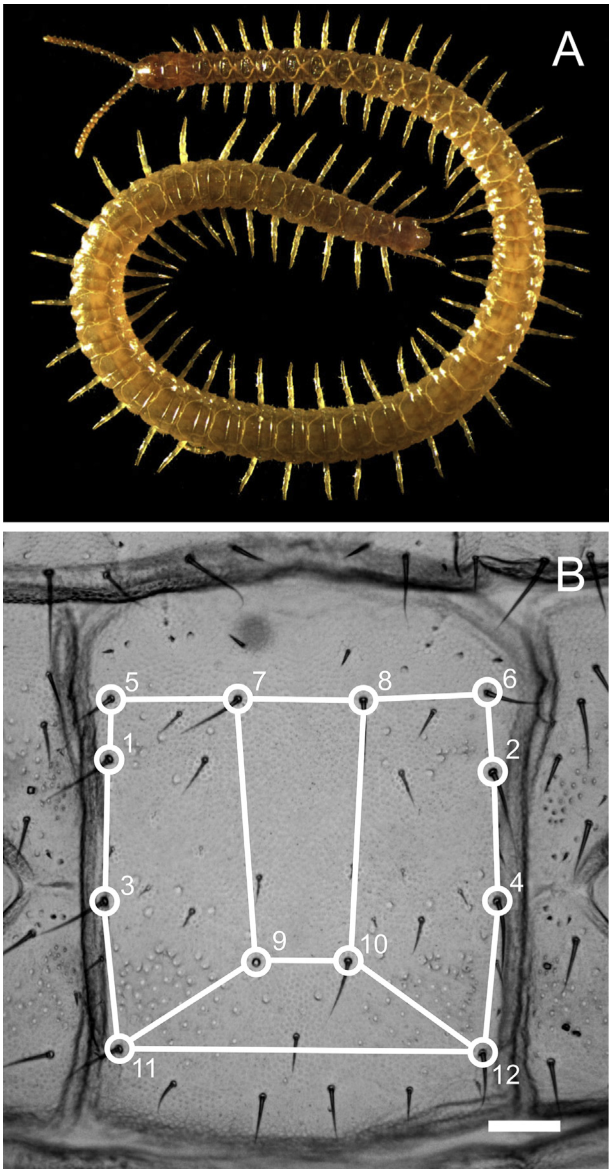


Fig. 1. The geophilomorph centipede *Strigamia maritima*. **A.** A 49 leg-pair female in dorsal view. The specimen is about 3 cm long (credit C. Brena). **B.** Landmark configuration on ventral metasternites adopted in this study (here the metasternite of leg-bearing segment 36 of specimen 8). The 12 landmarks are positioned at the basis of as many idionymic setae (see text). Scale bar 0.1 mm.

maxillipedes (the forcipules), a number of segments bearing one pair of legs each, and a short terminal apodous ano-genital region of uncertain segmental composition (Fusco and Minelli, 2013) (Fig. 1A). In geophilomorphs, the number of leg-bearing segments varies among species and, in most species, also within species, and does not change during post-embryonic developmental stages (with the possible exclusion of the very first embryo-like post-embryonic stages; Brena, 2014). In *S. maritima* the number of leg-bearing segments varies mostly from 45 to 49 in males and from 47 to 53 in females (Kettle and Arthur, 2000).

Our morphometric analyses are based on a small sample of *S. maritima* adult specimens, a sample that is nonetheless exceptionally homogenous for a geophilomorph species. It includes 9 adult specimens of the same sex (female), from the same locality (Deer Island, Galway, Ireland), and collected the same day (19.10.2007). Notably, all specimens have also the same number of leg-bearing segments (49, which was the modal number for the females of the locality and season, Eibner and Arthur, 2012).

The latter characteristic of this sample is particularly convenient for comparative morphometrics, as it allows excluding size and shape variation due to the development of the segmental pattern on a variable number of trunk segments. In fact, the morphology of segments appears to be related to their relative (rather than absolute) position within the trunk (Berto et al., 1997; Fusco and Minelli, 2000a). In our sample absolute (ordinal) and relative segmental positions of any given segment coincide. Moreover, uniformity in the collection date and locality contribute to limit environmental biases on size and shape variation, as both a longitudinal cline (Kettle and Arthur, 2000) and a temperature-dependent plastic effects (Vedel et al., 2008, 2010) on the number of leg-bearing segments have been described for the species.

The specimens are conserved in the Minelli-Bonato Chilopoda collection (Department of Biology, University of Padova, Italy), stored in 70% ethanol.

2.2. Sample preparation for microscopy

Preparation aimed at having all relevant ventral structures of the exoskeleton laying on the same plane.

Specimens, that were preserved in 70% ethanol, had the most anterior (head and forcipular segment) and the most posterior (last leg-bearing segment and the ano-genital region) portions of the body removed with a lancet and stored separately. After that, all the legs of the remaining trunk were cut at their basis with micro-scissors. Following this preparation, the trunk was treated with 10% KOH at 50 °C for 3 h. With this operation, all tissues were dissolved, leaving only the cuticular exoskeleton which was then washed in distilled water. Specimens were transferred from water to 100% glycerol through gradual transitions in solutions of increasing glycerol concentration until complete replacement. The resulting exuvia-like preparation was cut longitudinally with micro-scissors along the pleural regions of both sides, to get the series of ventral trunk sclerites loose. For microscopy sessions, these were semi-permanently whole-mounted on slides with their ventral side facing upwards, in glycerol.

2.3. Segment sampling and image acquisition

The treatment partially damaged the first and the last trunk segments, thus our study was restricted to the segmental series from leg-bearing segment 2 to 48. Another 7% of segments scattered through different specimens and segmental positions were excluded from the analysis because of local damage, so that sample size for specific segments varies between 5 and 9 specimens.

For each segment, we considered a single, non-articulated exoskeletal structure that is the largest, nearly flat, ventral sclerite called the *metasternite* (Fig. 1B). Photographs of each metasternite for all specimens were taken using a digital camera (Leica DFC 400) mounted on a light microscope (Leica DM LB). Images were acquired through the Leica application suite software (V.2.8.1) at a resolution of 2592 × 1944 pixels.

2.4. Landmark choice and data acquisition

Within each metasternite, we used as landmarks 12 idionymic sensory setae, i.e. setae that are serially homologous across trunk segments within an individual (Minelli and Fusco, 2013), and homologous across individuals within species (Fusco and Minelli, 2000a) (Fig. 1B). The selected setae are the most recognizable ones. In *S. maritima*, setae appear progressively on the metasternite in a stereotypic succession during post-embryonic development

(Horneland and Meidell, 2009) and their size and recognisability in adults is proportional to the number of developmental stages (or moults) that have elapsed since their first appearance. All the selected setae are present since the first post-embryoid stage (*adolescens I*; Brena, 2014).

In general, the spatial localization of the setae is indicative of the size and shape of the sclerite bearing them, however for the finer effects of size and shape FA it must be noted that their configuration more precisely reflects the topology of the underlying peripheral nervous system (sensory system).

To assess measurement error due to digitizing, landmarks were digitized twice by the same operator (MB), in two independent working sessions on different days, using TPSDig 2 (ver. 2.17; Rohlf, 2015). The program tpsUTIL (ver. 1.60; Rohlf, 2015) was used to build the final (NTS) data files and combine them into a single dataset.

2.5. *Morphometric analyses*

2.5.1. *Size analyses*

We used both traditional and geometric morphometric estimates of size. Ordinary length and width for each segment were obtained by averaging the distances between landmarks 5–11 and 6–12 for the length, and 1–2 and 3–4 for the width (Fig. 1B). Centroid size is calculated as the square root of the sum of the squared distances of all landmarks from their centroid (Slice et al., 1996). To account for overall size differences among specimens, segment size measures of a specimen were normalized by dividing them by the average segment centroid size in the specimen in the segmental interval 21–38 (where there are no missing data for any specimen). Such normalized size measures were finally averaged across specimens and replicates to obtain an estimate of the segmental pattern for the species.

2.5.2. *Shape analyses*

Each leg-bearing segment is bilaterally symmetric and all segments are also arranged following translational symmetry. Therefore, the anatomical organization of these organisms reveals a composite pattern of bilateral and translational symmetry.

In this study, we primarily focus on the variation in bilateral symmetry along the segmental series. Each segment possesses an axis of bilateral symmetry that runs through the structure itself and separates the left and right sides as two connected halves that are mirror images of each other. This specific type of symmetry can be handled within the morphometric framework of *object symmetry* (Mardia et al., 2000; Kent and Mardia, 2001; Klingenberg et al., 2002; Savriama and Klingenberg, 2011). Following this approach, the original configuration of landmarks consists of paired landmarks that are mirror images of each other relative to the axis of symmetry and are located outside of it, while unpaired landmarks are aligned on the axis of symmetry (no unpaired landmarks in our case). First, the original configurations of landmarks were duplicated, and then, these copies were reflected with a suitable relabelling of the paired landmarks that consists in mutually exchanging the labels of each corresponding symmetric pair. Thereafter, separate Generalized Procrustes Analyses (GPA) were applied to each segment (i.e., each doubled dataset) in order to remove extraneous information of size, location and orientation, and extract shape data according to a least squares criterion (Rohlf and Slice, 1990; Dryden and Mardia, 1998). In the context of object symmetry, the shape variation extracted from each segment can be decomposed into two components, the symmetric and asymmetric components of shape variation (Mardia et al., 2000).

We used the symmetric component to characterize the among-individual variation and summarize the main pattern of shape variation using Principal Component Analysis (PCA). We calculated the average segment shape and also modelled the average symmetric variation in shape along the body axis with multivariate polynomial regression of Procrustes shape variables on centroid size (degree 5 polynomial). Predicted average shapes for each segment and the regression curve were superimposed onto the shape space to illustrate the average pattern of segment heteronomy.

For the analysis of bilateral asymmetry, we carried out the two-way mixed model Procrustes analysis of variance (ANOVA) traditionally used in studies of fluctuating asymmetry. For each segment, the total shape variation was decomposed into the main effect of ‘individual’ (i.e. variation among individuals – symmetric component), ‘side’ (i.e. directional asymmetry – non-random variation between the two sides), the interaction ‘individual-by-side’ (i.e. random variation within individuals – FA) and measurement error due to digitizing (Leamy, 1984; Palmer and Strobeck, 1986; Klingenberg et al., 2002). The covariance matrices associated with each of these effects were also used to visualize and compare patterns of covariance. It is important to note that in the context of object symmetry, the symmetric and asymmetric components of shape variation occupy orthogonal subspaces of the shape space (Mardia et al., 2000; Kent and Mardia, 2001; Kolamunnage and Kent, 2003). Following the recommendation of Klingenberg et al. (2002), we therefore performed the comparison of covariance structures using only the parts of the covariance matrices corresponding to half-configurations (there was no need to exclude unpaired landmarks since our morphometric scheme does not include any). We specifically focused on two covariance matrices: one corresponding to the individual variation and one to FA. These matrices respectively describe the patterns of segmental variation among individuals and the patterns of variation between left and right sides (i.e. variation within individuals that represents the stochastic part of variation). For each segment, comparing these two matrices allowed us to investigate whether individual variation (canalization) and bilateral FA (developmental stability) are concordant. Across segments, comparing these matrices provides a means to assess whether the shape variation is dependent on the segment position, or rather, is stable all along the body. All 94 matrices (47 segments with each two associated covariance matrices, for individual variation and FA, respectively) were compared by first computing their pairwise dissimilarities (as one minus their correlation values), and then carrying out a Principal Coordinate Analysis (PCoA) on the dissimilarity matrix (see for example Debat et al., 2006; Mitteroecker and Bookstein, 2009 for similar use), to graphically display the relationship among these matrices.

Additionally, we include an analysis of translational FA following the procedure described in Savriama et al. (2016). For this analysis, we used the segmental interval numbered 12–20 (nine segments) that corresponds to the region of the trunk investigated in Savriama et al. (2016).

All analyses were programmed and carried out in R (R Core Team, 2016) and in MorphoJ (Klingenberg, 2011).

3. Results

3.1. *Segmental patterns of size and shape*

The segmental pattern of centroid size shows an approximately parabolic profile (Fig. 2A). This closely resembles that of the width of the segments, while segment length, with the exclusion of the extreme anterior and posterior elements of the series, exhibits a flatter trend (Fig. 2B).

Metasternite shape variation along the trunk shows a curvilinear trend, marked by a strong bent around segments 24–32 (Fig. 3). Segments shape along the body axis is at first narrow

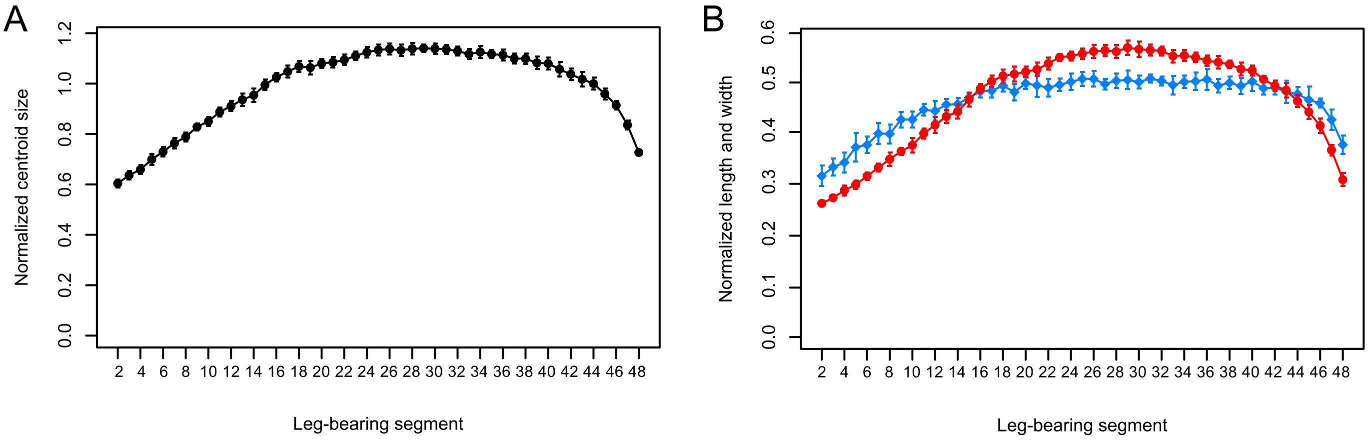


Fig. 2. Pattern of metasternite size variation along the body in *Strigamia maritima*. **A.** Centroid size. **B.** Length (diamonds) and width (dots). Bars are standard deviations.

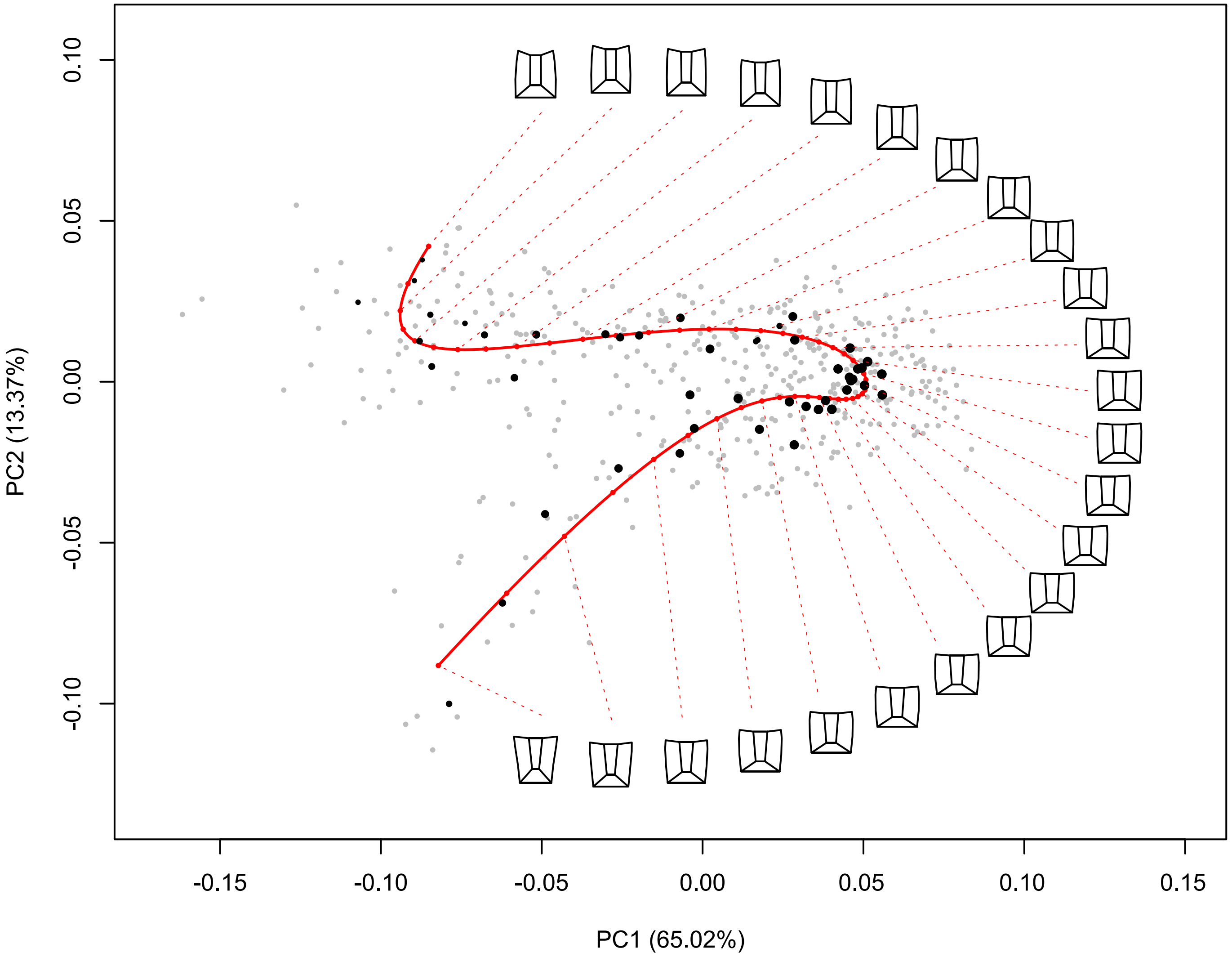
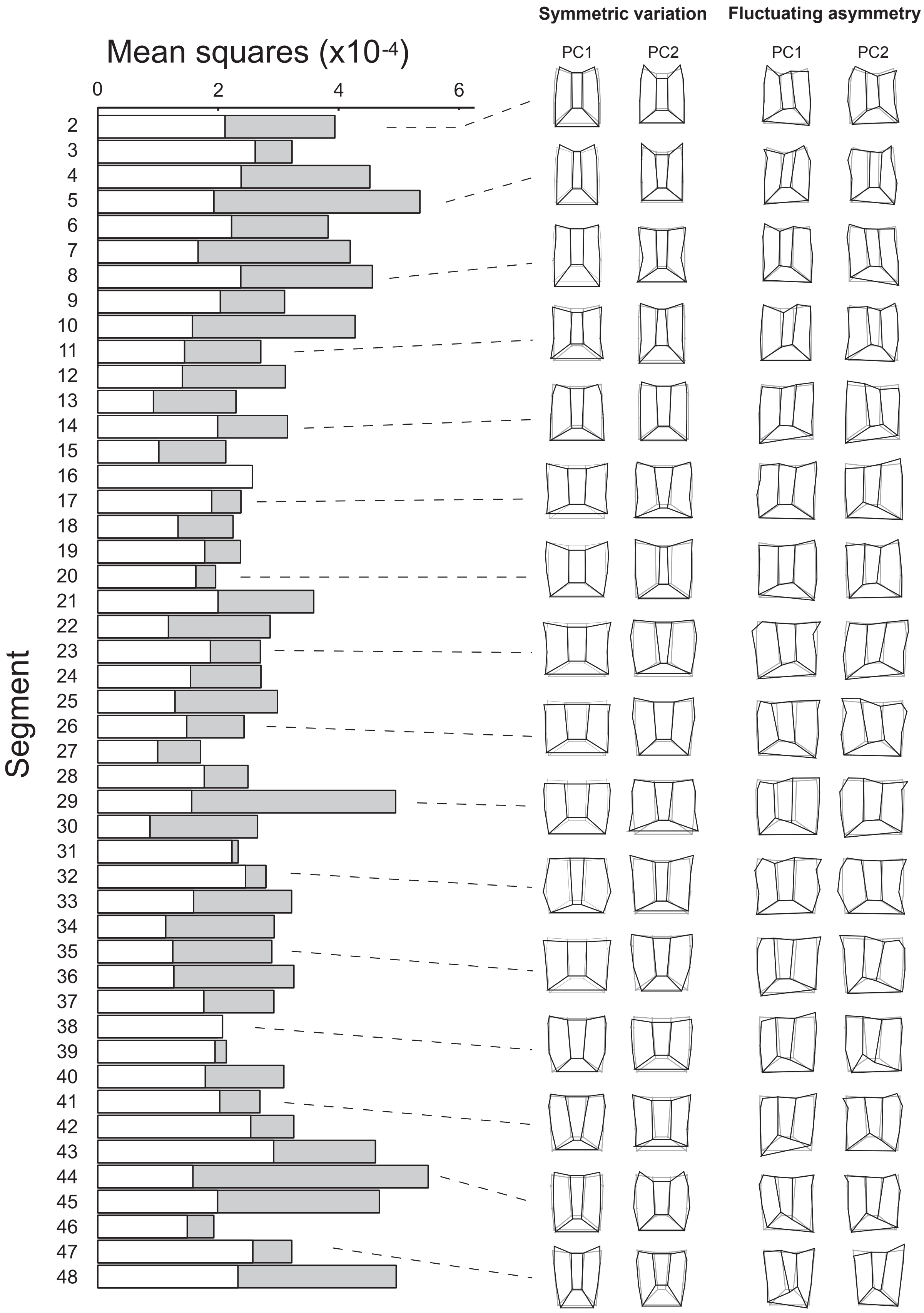


Fig. 3. Shape space of metasternite variation in *Strigamia maritima*. Grey dots are individual segments of each specimen. Black dots are segment average shapes and their size is proportional to centroid size. The curve line is a multivariate polynomial regression of shape on centroid size. Wireframes of metasternite predicted shapes are shown every two segments.


Symmetric variation
Fluctuating asymmetry
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PC2
PC1
PC2
Mean squares (x10-4)
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Segment
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bottom-heavy trapezoidal, then it becomes more rectangular in the midregion, to end with a top-heavy trapezoidal shape.

3.2. Individual variation and bilateral asymmetry in shape along the trunk

The levels of shape individual variation and FA measured by mean squares (i.e. variances) obtained from two-way mixed model Procrustes ANOVAs (Fig. 4), indicate that the mean squares for the individual variation vary from $1.7\cdot10^{-4}$ to $5.5\cdot10^{-4}$ (average: $3.2\cdot10^{-4}$, s.d: $1.0\cdot10^{-4}$) and spread for FA from $0.9\cdot10^{-4}$ to $2.9\cdot10^{-4}$ (average: $1.8\cdot10^{-4}$, s.d.: $0.5\cdot10^{-4}$). Interestingly, the main effect of individual variation compared to FA is not significant for one-third of the segmental series (segments 3, 9, 16, 17, 19, 20, 23, 28, 31, 32, 38, 39, 41, 42, 46 and 47) (see Section 4.2).

No clear trend emerges from the levels of individual variation and FA along the body axis, but for a weak but significant correlation between the mean squares of individual variation and FA (correlation = 0.347; P-value = 0.017; $N = 47$), suggesting that the most variable segments tend to be the most developmentally unstable as well. The patterns of morphological variation described by PCA for the individual variation and FA do not show any distinct tendency along the body axis, although the anterior part of the sclerite (landmarks 1, 2, 5, 6, 7 and 8) seems to vary in an independent manner compared to the posterior one (landmarks 3, 4, 9, 10, 11 and 12) within segments (Fig. 4).

The Principal Coordinate Analysis (Fig. 5) provides a multivariate ordination of the covariance matrices associated with the individual variation and FA. It shows a clear separation of the two sets of matrices, regardless of the segment considered. Nevertheless, individual variation and FA matrices share similar features: anterior-most and posterior-most segments (2–11 and 46–48) exhibit patterns of variation that are more similar with each other than compared to the rest of the segmental series (12–45).

The mixed-model Procrustes ANOVA for translational FA indicates that the ‘individual’ main effect (i.e. variation among individuals), the ‘segment’ main effect (the average deviation from one segment to the segments' mean), the interaction term ‘individual-by-segment’ (i.e. the variation, among individuals, in the heterogeneity among segments) are all significant (not shown here). The levels of translational FA measured as mean squares for size and shape from the interaction term ‘individual-by-segment’ are $2.1\cdot10^{-4}$ and $1.7\cdot10^{-4}$, respectively.

4. Discussion

4.1. Segmental patterns of size and shape in Strigamia

Size segmental patterns (Fig. 2) are to a large extent similar to those reported for other geophilomorph species (Berto et al., 1997; D. Berto, unpublished data). However, they are more strictly lacking any discontinuities or localized abrupt slope changes than other geophilomorph species (e.g., *Clinopodes flavidus*, Fusco and Minelli, 2000a).

Shape segmental pattern show a marked bend at about 60% of the leg-bearing trunk. This point does not correspond to either i) the position along the trunk where *S. maritima* embryo and postembryo is bent within the egg shell, from late embryonic (stage 6) to early postembryonic (proembryoid III) stage, which is approximately at 35% (Brena, 2014); ii) the position of the so-called mid-body transition, that in many geophilomorph species marks the divide between an anterior and a posterior set of segments, differing for instance in leg size or for the presence of species-specific morphological features on the trunk sclerites, which is generally described to occur at about 40% of the trunk (Minelli and Koch, 2011); or iii) any boundary of Hox gene expression, as *S. maritima* Hox genes do not exhibit either anterior or posterior boundaries in their embryonic expression domain within the leg-bearing trunk (Brena, 2015).

So far, there are no similar data available from other centipedes to compare with. However, curiously, the shape segmental pattern in the PCA plot (Fig. 3) resembles the one observed in a multivariate regression of shape onto centroid size for the vertebral series of a viperid snake (*Daboia russelli;* Sarris et al., 2012). This similarity might also stem from an allometric effect, as both anterior and posterior segments are smaller than the median ones. In both species, the similarity between the anterior and the posterior ends of the segmental series contrasts with the mechanism of embryonic segmentation, which both in centipedes and vertebrates is sequential, form anterior to posterior (Brena, 2015; Dequéant and Pourquié, 2008). This indicates that positional specification in segmental patterning is not strictly associated to the timing of embryonic segment addition.

4.2. Shape asymmetry and variation along the trunk in Strigamia

Although it is difficult to detect a pattern in the distribution of the magnitude of individual variation along the trunk, as shown in Fig. 4 (i.e. no particular region is markedly more variable than another), it is nevertheless clear that segments do not vary to the same extent. Interestingly, the shape FA and shape variation among individuals are correlated among segments, meaning that the most randomly asymmetric segments (i.e. the less developmentally stable ones) also tend to be the most variable among individuals (the less canalized ones). This correlation should be considered cautiously, as the variances were estimated on nine individuals only. Nevertheless, it suggests either that canalization and developmental stability are not independent from one another, or that their efficiency is jointly and similarly affected by the position of the segment along the body.

The main effect of individual variation compared to FA is not significant for one-third of the segmental series. One should expect more variation among individuals and less variation within individuals, in the form of variation between left and right sides, since in motile bilaterally symmetrical organisms the two sides on average experience the same environmental influences. The modest difference between individual variation and FA recorded in *Strigamia* is instead more similar to that observed in some sessile organisms, where the two sides, or a set of symmetric body parts, can be consistently exposed to differential environmental conditions, and their variation can be greater than the variation across individuals (Savriama and Klingenberg, 2011; Savriama et al., 2012; Neustupa, 2013, 2017).

The Principal Coordinates Analysis separates the matrices of individual segment variation and those of FA clearly, regardless of the segment considered. This shows that the variation of a segment shape among individuals is to a certain degree different from its variation between body sides. This would be therefore indicative of a difference in the processes ensuring canalization and developmental stability.

Fig. 4. Shape variation and asymmetry along the trunk of *Strigamia maritima*. Left: Barplot showing the levels of individual variation (symmetric component – grey bars) and within-individual variation (FA – overlapping white bars) represented by mean squares (i.e., variances) derived from two-way mixed model Procrustes ANOVAs. Right: Principal components describing the patterns of morphological variation for individual variation and FA. The differences between the wireframe configuration with the black dots and black lines and the configuration with the grey dots and grey lines (average shape) represent the shape change for a Procrustes distance of +0.1.

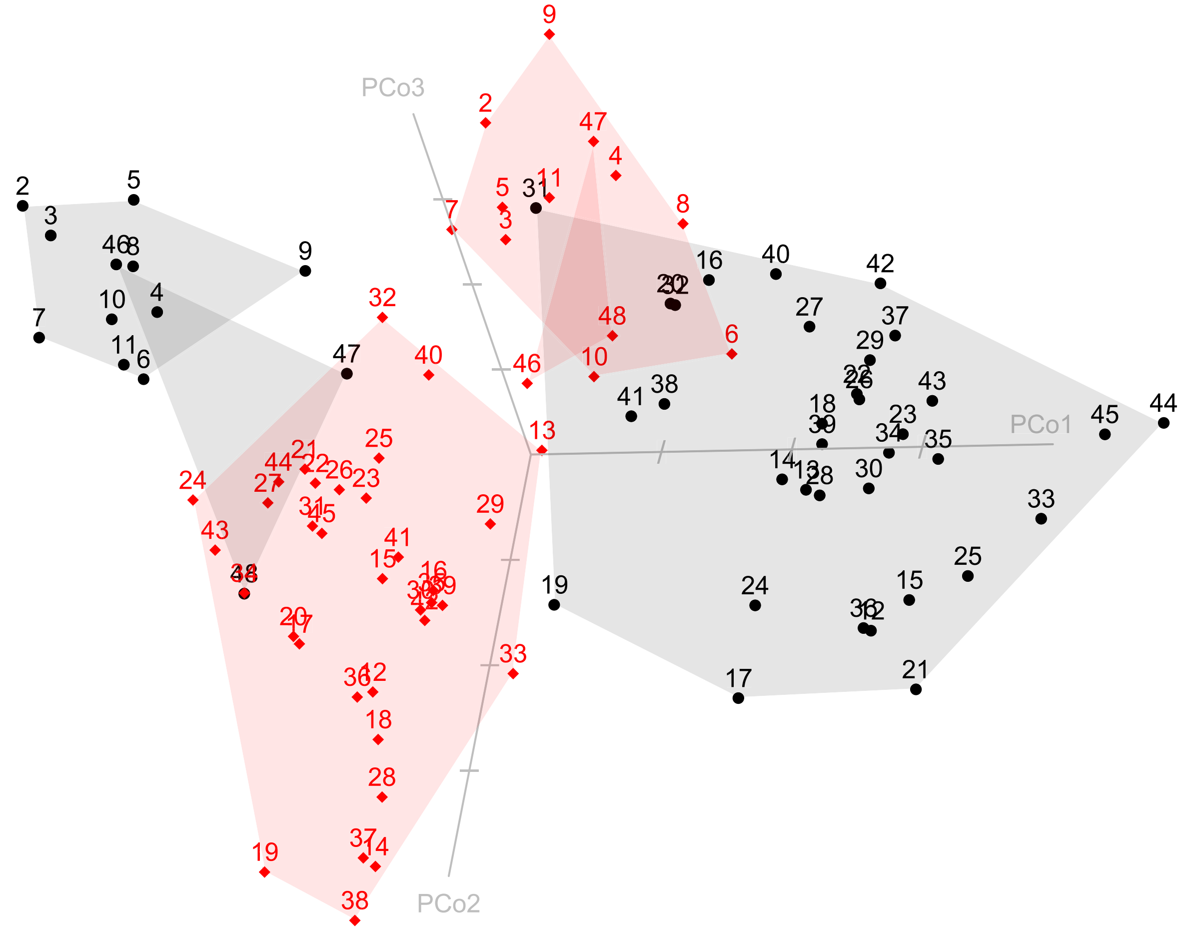

Fig. 5. Principal Coordinate Analysis (PCoA) of the shape covariance matrices associated with the individual variation and FA. Dots represent the matrices for individual variation. Diamonds stand for the FA matrices. Labels indicate segment position along the series. Polygons highlight anterior (2–11), central (12–45) and posterior (46–48) groups of segments (see text).

In contrast, there is a clear opposition of the patterns of shape individual variation of the 11 most anterior segments (2–11), together with those of the three most posterior ones (46–48), from all the others, more central segments (12–45). This is remarkably similar for the patterns of shape asymmetry, opposing the exact same segments. This suggests (i) that the patterns of variation are clearly dependent on the segment position along the body. This might be related to the antero-posterior shift in segment shape described in Fig. 3, which suggests a somewhat similar shape for the first and last segments. However, as both individual variation and asymmetry are similarly affected by the segment position, this also suggests (ii) that both canalization and developmental stability are specific of – and dependent on – the segment considered. This could be interpreted in two ways: (1) it might indicate that there would be no such thing as an individual-wide control of phenotypic variation, but rather local processes buffering variation at the level of each individual segment. Alternatively, (2) some global buffering processes might be at play, but their effect might be modulated locally.

There is thus some evidence that canalization and developmental stability are not independent from one another, as some degree of similarity is detected, in that both the amount of individual variation and FA, and their patterns of phenotypic expression (shape) are affected by the position of the segment along the body. However, our results do not allow us to firmly conclude whether canalization and developmental stability i) share the same developmental basis, or ii) are similarly influenced by the position along the body. Besides, the clear distinction in the PCoA, of the patterns of individual variation and asymmetry suggests at least some developmental differences.

Levels of translational FA in shape and size in *S. maritima* are 10 times lower than those observed in other eight geophilomorph species (Savriama et al., 2016). However, these results might not be straightforwardly comparable, as the sets of landmarks in the two studies are different, and it is not obvious how to trace homologies even among subsets of the two. Also, different techniques for image acquisition were used. The analysis of translational FA is a recent addition to the GM techniques for the study of FA, and beyond refinement statistical treatment (see Section 4.3), also more exploratory studies on the side of data acquisition are needed.

As a conclusive remark, it should be restated that data collected from only nine individuals were used, and therefore we should be cautious with the results provided by the statistical tests and their interpretation. More data need to be collected and further analyses need to be conducted to get to more firm conclusions.

4.3. *Geometric morphometrics and segmental patterning*

Arthropod segmentation embraces a multiplicity of developmental processes, that span from gene expression, to epithelial morphogenesis, to allometric growth (Fusco, 2005). Segmental patterning, is a specific aspect of segmentation, that focuses on segment differentiation, but it is in no way a follower of segmentation or a distinct developmental process.

Here we have shown how GM analyses of size and shape variation can provide information on key aspects of segmental patterning. These techniques can offer detailed descriptive data for comparative purposes, but can also provide precious information on some features of the developmental system that are considered relevant for the evolvability of a segmented body architecture. In an evo-devo perspective, the study of developmental stability and canalization, as well as the dissection of several sources of phenotypic variation along a segmented body axis, like constitutive and random segment heteronomy, and the investigation of their interactions, both within and among individuals, are of the highest interest for an evolutionary approach to segmentation. Moreover, the result of these kind of analyses can be directly compared with developmental genetic data, like the expression domain of Hox genes in bilaterians (Head and Polly, 2015).

While most of the analyses presented here (e.g., PCoA) have been devised since long, and their value (as well as their limitations) have been examined in depth by the morphometrician community (e.g., Debat et al., 2006; Mitteroecker and Bookstein, 2009), other analyses, like the association of canalization and developmental stability and the analysis of translational FA, are either more contentious or much more recent and have been tested in a smaller number of studies (e.g., Nuche et al., 2014).

GM approaches are promising for studying the variation of the developmental buffering across body segments, as it allows to investigate simultaneously the patterns of shape variation of all segments of all individuals at once. Comparing populations differing by their genetic and/or environmental conditions might be interesting in this context. Interspecific comparisons would be very interesting as well, as patterns of heteronomy might have different effects across species.

For the study of translational FA, it should be noted that some aspects of our study design, although sensible for the specific case, do not represent a general solution for the treatment of translational FA. A first issue is how to tease apart translational FA from constitutive segment heteronomy, as i) the elements of a segmental series are in general not expected to be all perfectly identical to each other, and ii) different individuals can have distinct constitutive segmental pattern (target phenotype). Thanks to the modest segmental differentiation in geophilomorphs and the shortness of the trunk segmental series we used for this analysis (nine segments) we could approximately separate constitutive from fluctuating translational asymmetry by applying a linear correction (individual-specific linear regression, see Savriama et al., 2016). But this is not a general solution, as segmental patterns can be more complex, especially if evaluated on longer segmental series. A second issue concerns the comparison of segmental series of different length. In this study and in Savriama et al., 2016 we used a sequence of segments at 1/3 of the trunk, irrespective of the total number of segments, because previous studies (e.g., Berto et al., 1997) showed that, for geophilomorph species, the relative position of the segments along the trunk, rather than their absolute position, appears to be the major determinant of their size and shape. However, the question of the homology of segments has no simple and univocal solution for all organisms. Only moving to another, centipede, taxon, the Scolopendromorpha, one can observe that some anatomical features exhibit absolute (segmental) positional homology, while others have relative (segmental) positional homology (Fusco and Minelli, 2013). There seems to be no general solution to this question, and a careful preliminary evaluation of homology relationships is a fundamental requirement for sound comparative studies. Moreover, the relationship between bilateral and translational fluctuating asymmetries in segmented organisms, is almost unexplored (Fusco and Minelli, 2000b; Nuche et al., 2014; Savriama et al., 2016).

At least some of the questions raised for translational symmetry in these segmented animals apply to other segmented organisms and to other forms of symmetry as well. Heteronomy, the differentiation among homologous body parts, in all its forms, is not just a technical difficulty for the study of organismal symmetry. On the contrary, it invites to a more profound understanding of symmetry, in particular in relation to the developmental processes that generate it, and shows the necessity of a developmentally-based approach to the study of the evolution of organismal body architectures.

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