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Anaesthetization and fixation effects on the morphology of sabellid polychaetes (Annelida: Polychaeta: Sabellidae)

Elisa Maria Costa-Paiva*, Paulo Cesar Paiva and Michelle Klautau

Departamento de Zoologia, Instituto de Biologia, Bloco A, CCS, Universidade Federal do Rio de Janeiro, Ilha do Fundão, 21941-590, Rio de Janeiro, RJ, Brazil. *Corresponding author, e-mail: elisapolychaeta@hotmail.com

A hundred and four specimens of *Branchiomma luctuosum* (Grube, 1870), a sabellid polychaete, were submitted to different anaesthetization and fixation procedures: a control group with living animals, immediate fixation with formaldehyde 4% (FO), ethanol 100% (AE) or ethanol 70% (ET), and anaesthetization with magnesium chloride (MC), refrigeration (RE), menthol crystals (ME) or freshwater (FW). Twenty-three morphometric variables of the body were measured and analysed with size-free multivariate statistics. The first three canonical variables explained 80% of the variation, being significantly correlated with 19 measured variables, mainly those related to the shape of the body setigers. Besides morphometric variables, some morphological characters commonly used in polychaete taxonomy also presented variation among fixation procedures, such as the release of parapodial elements and the integument. Among the procedures tested, AE, ET and FW were the best in approximating the shape of living specimens. Specimens submitted to FO and RE had shorter, wider, and thicker bodies, whereas those submitted to MC and ME were more elongated, narrower, flatter, and had wider pygidia than living specimens. Lengths of branchial crown, setiger 1 and pygidium seem to be the most informative morphometric characters for taxonomic purposes since they did not present deformation due to fixation procedures.

INTRODUCTION

Systematists are often interested in quantifying morphological differences among species, conspecific populations, or ontogenetic stages (Strauss & Bookstein, 1982). Body shape represents an important set of animal characters that are used for several purposes, such as studies in taxonomy, ecology, evolution, growth and morphological abnormalities (Rohlf & Marcus, 1993; Lestrel, 2000). Therefore, morphometrics became a fundamental tool to assess shape and shape variation in biology (Rohlf, 1990).

Numerous taxonomic studies have used multivariate morphometrics successfully to analyse differences among species and even among populations (e.g. Debuse et al., 2001; Jordaens et al., 2002; O'Reilly & Horn, 2004). However, there are few morphometric studies on polychaete worms, such as Mackie (1984), Fauchald (1991), Sigvaldadóttir & Mackie (1993) and Martin et al. (2003). For soft-bodied animals or structures, body shape depends on the degree of relaxation (Gustus, 1972). Hence, the measurements of some soft-bodied animals or structures after fixation may not reflect their real shape, due to deformation. Consequently, morphometric differences observed among groups of animals submitted to different methods of fixation and/or anaesthetization could be merely methodological artefacts (Howe, 2002).

The effects of fixatives on body shape are difficult to predict due to variability related to the type and concentration of fixative, life stage, life habit and other factors (Sagnes, 1997). Methodological artefacts owing to anaesthetisation and fixation procedures are noticeable when comparing material from different origins, such as museum and fresh material. Even for museum specimens, the lack of information concerning anaesthetization and prior fixation methods could restrict comparisons among specimens when shape information is necessary.

Experimental studies on potential morphological modifications related to methods of fixation are still scarce for most animal groups (Fowler & Smith, 1983; Kruse & Dalley, 1990; Quiñonez-Velázquez & Chaumillon, 1996; Sagnes, 1997; Jordaens et al., 2002) and absent in polychaetes. Mostly, studies regarding anaesthetization methods are more engaged in the maintenance of living animals for transportation rather than on shape recovery of dead or fixed animals (Heasman et al., 1995; White et al., 1996; Bower et al., 1999).

In the present study, the effects of different methods of anaesthetization and fixation on morphometric variables were assessed in the polychaete marine worm *Branchiomma luctuosum* (Grube, 1870). *Branchiomma luctuosum*, a species of the family Sabellidae, is a sedentary and tubiculous species frequently associated with hard surfaces (Rouse & Pleijel, 2001). The aims of our study were three-fold: (i) to determine which is the most appropriate fixation method to preserve the shape of living *B. luctuosum;* (ii) to describe how different anaesthetization and fixation treatments affect morphological variables; and (iii) to define the most informative morphometric characters for taxonomy, which are the less affected by methodological fixation procedures.

Table 1. *Variables measured for morphometric analysis and their abbreviations.*

	Morphometric variables	Landmark definitions and remarks
TL	Total length without branchial crown	measured from the collar to the end of pygidium
BC	Length of branchial crown	measured from the tip of the longest radiole to the base of branchial lobe
TH	Length of thorax	measured from the collar to the last thoracic setiger
LS1	Length of setiger 1	
DS1	Depth of setiger 1	measured with the specimen laid in a lateral position
WS1	Width of setiger 1	
LS4	Length of setiger 4	chosen because it represents the middle of the thorax
DS4	Depth of setiger 4	measured with the specimen laid in a lateral position
WS4	Width of setiger 4	
LS8	Length of setiger 8	chosen because it is the last thoracic setiger
DS8	Depth of setiger 8	measured with the specimen laid in a lateral position
WS8	Width of setiger 8	
LS9	Length of setiger 9	chosen because it is the first abdominal setiger
DS9	Depth of setiger 9	measured with the specimen laid in a lateral position
WS9	Width of setiger 9	
LS20	Length of setiger 20	
DS20	Depth of setiger 20	measured with the specimen laid in a lateral position
WS20	Width of setiger 20	
LS50	Length of setiger 50	
DS50	Depth of setiger 50	measured with the specimen laid in a lateral position
WS50	Width of setiger 50	
LP	Length of pygidium	
WP	Width of pygidium	

MATERIALS AND METHODS

Sampling

Specimens of *Branchiomma luctuosum* were sampled by snorkelling at a depth of 3 m on 9 July 2003 at Urca beach, Rio de Janeiro State, on the south-eastern Brazilian coast (22°57.1'S 43°09.9'W). Specimens were transferred to a seawater container. Only one field survey was done to avoid spatial and/or temporal variation that may have affected our results and conclusions.

In the laboratory, the specimens were withdrawn from their tubes and selected for posterior experimental procedures; juveniles and specimens under regeneration were excluded. Thus, only adults were kept alive in a controlled temperature container (18°C–20°C), in an attempt to prevent reactions caused by thermal stress.

Experimental procedures

A total of 104 specimens were analysed, 13 being randomly submitted to each experimental treatment. Procedures were chosen according to commonly used fixation methodologies for polychaetes or invertebrates in general (e.g. Fauchald, 1977; Lincoln & Sheals, 1979; Amaral & Nonato, 1987; Rouse & Pleijel, 2001). Except for the refrigeration procedure, all the procedures were conducted at a constant room temperature of 20°C. The following procedures were used:

(1) Previous anaesthetization—living specimens were completely relaxed with the following anaesthetics being further fixed in 4% formaldehyde. Anaesthetization time, for each procedure, was defined based on previous empirical observations for this species:

(a) Magnesium chloride (MC)—for 30 minutes in a solution isotonic to seawater (7.5 g $MgCl_2$.$6H_2O$ in 100 ml of distilled water);
(b) Refrigeration (RE)—for eight hours, specimens were put individually into containers with seawater which were incubated inside another container with ice, where the temperature varied from 1°C to 4°C;
(c) Menthol crystals (ME)—for three hours with a few crystals previously added to seawater;
(d) Freshwater (FW)—for 20 minutes, in 1 l.

(2) Fixation without previous anaesthetization—specimens fixed for 10 d in:

(a) Formaldehyde 4% (FO)—the stock solution was diluted in seawater;
(b) Ethanol 100% (AE);
(e) Ethanol 70% (ET)—ethanol diluted with regular tap water.

A control group (CO) of living specimens was maintained in a container with seawater during the study.

Data collection and analysis

For each specimen 23 morphometric measurements were taken (Table 1; Figure 1) with a graduate stereoscopic microscope (ZEISS Stemi SV11). The morphometric variables were measured in millimetres and subsequently normalized by a logarithmic transformation (Sokal & Rohlf, 1995). The effect of size was previously removed by regressing each variable on the first principal component (PC1) achieved by means of a principal component analysis (PCA) (Humphries et al., 1981). Residuals obtained by such regression are likely to represent shape variation since,

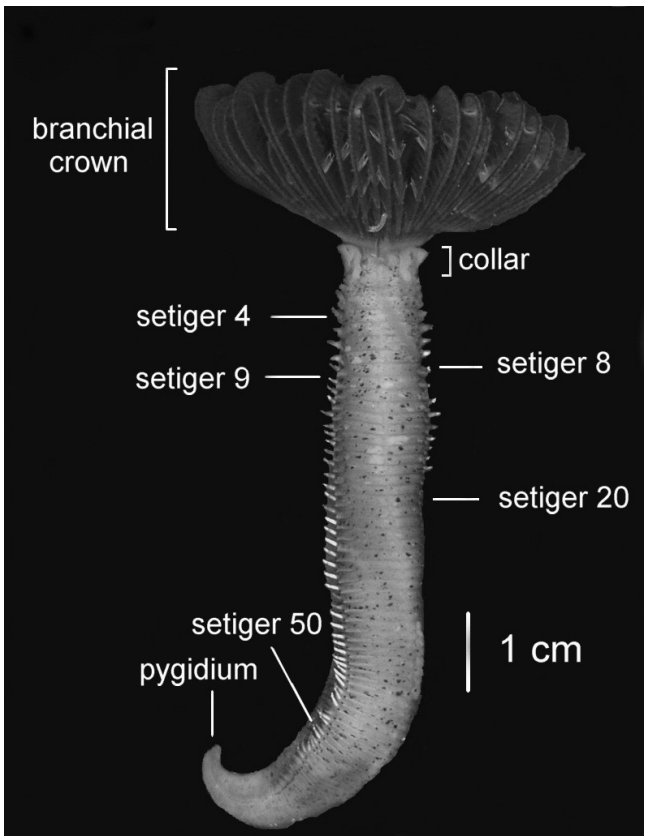

Figure 1. Picture of a living specimen of *Branchiomma luctuosum* showing the characters that were measured.

in this case, PC1 can be considered as a multivariate size estimator (Marcus, 1990).

Residuals were used in canonical discriminant analysis (CDA) and a multivariate analysis of variance (MANOVA) was applied in order to test the significance of shape differences among treatments. An analysis of variance (ANOVA) was applied for canonical variables to test the significance of observed variation among treatments, being followed by *a posteriori* Tukey's test (Sokal & Rholf, 1995). The relative importance of each variable in discriminating treatment procedures was assessed by linear correlations (Pearson's product-moment correlation) between the individual scores for each canonical variable (CV) and the individual residual value of each morphometric variable. Significance levels for individual correlations (P<0.05) were adjusted by the Bonferroni correction (Sokal & Rohlf, 1995). All multivariate analyses were performed using SYSTAT® v. 10.0 (SPSS Inc., Chicago) software procedures.

RESULTS

Multivariate discrimination among treatments

In the size-free CDA, the first canonical variable (CV1) explained 50% of the variation among groups, the second (CV2) was responsible for 17%, the third (CV3) for 13%, and the remaining CVs (4 to 7) for 20%. Discrimination among treatment procedures was highly significant (Wilks' λ=0.004, $F_{[154,513]}$=4.23, P<0.0001). The percentages of correct assignment of individuals to original treatments were 69% (AE), 77% (ET and CO), 92% (FO, FW and MC) and 100% (ME and RE) with a mean value of 88%.

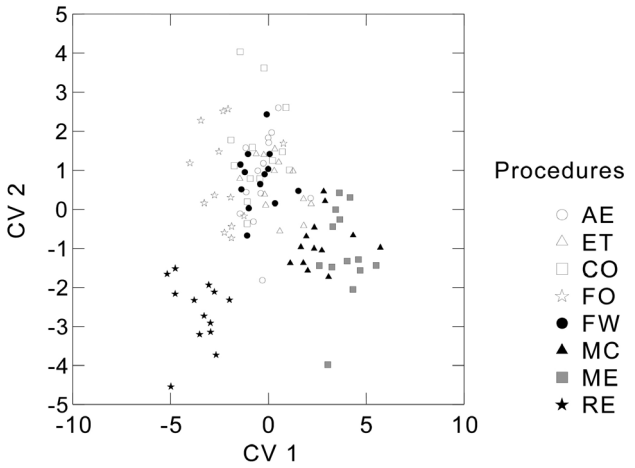

Figure 2. Plot of size-free canonical variables 1 and 2 (CV1 and CV2). AE, ethanol 100%; ET, ethanol 70%; CO, control; FO, formaldehyde 4%; FW, freshwater; MC, magnesium chloride; ME, menthol crystals; RE, refrigeration.

The plot of CV1 and CV2 showed a notable discrimination among procedures for both variables (Figure 2). Along the first canonical axis (CV1), treatments were significantly discriminated by ANOVA ($F_{[7,96]}$=75.17, P<0.001) in three main groups: (a) a group formed by procedure RE (anaesthetization by refrigeration) with the lowest scores; (b) a group formed by procedures MC and ME (anaesthetization by magnesium chloride and menthol, respectively) with the highest scores; and (c) a group with intermediate scores formed by CO (control), AE (ethanol 100%), ET (ethanol 70%), FW (anaesthetization by freshwater), and FO (formaldehyde 4%), the latter presenting significantly lower scores.

The second canonical variable differentiated significantly (ANOVA: $F_{[7,96]}$=25.58, P<0.001) among three main groups: (a) a group represented by procedure RE, with the lowest scores; (b) a group formed by CO, AE, ET, FO, and FW with the highest scores; and (c) a group with intermediate scores formed by MC and ME treatments.

Along CV3, a gradient was observed among treatments that could be gathered in three significant main groups (ANOVA: $F_{[7,96]}$=19.98, P<0.001) as shown in Figure 3: (a) a group formed by CO, RE, FW, ET, and MC with lower scores; (b) a group represented by procedure FO presenting the highest scores; and (c) an intermediate group formed by AE and ME.

Shape variation among treatments

From a total of 23 morphometric variables measured, CV1 was significantly correlated to 19 variables (P<0.05), CV2 to four, and CV3 to three (Table 2). Total length without branchial crown (TL), length of thorax (TH), and length of setigers (LS) 4, 8, 9, 20, and 50 were positively correlated to CV1, whereas the depth (DS) and width (WS) of all measured setigers were negatively correlated to CV1. Specimens submitted to treatments RE and FO presented lower values of variables TL, TH and LS (setigers 4, 8, 9, 20 and 50) and higher values of DS and WS; they were shorter, wider and thicker than living specimens (CO). On

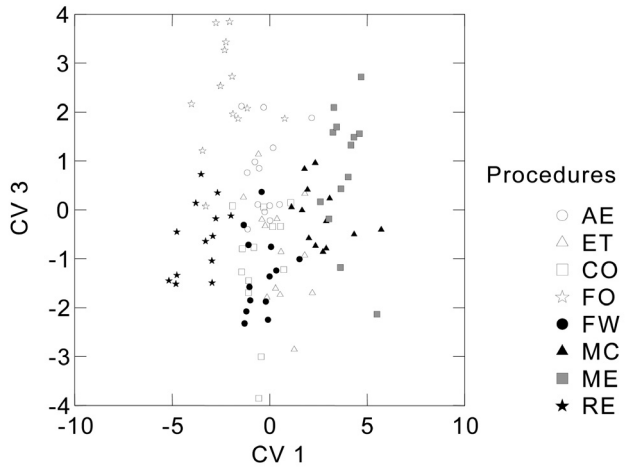

Figure 3. Plot of size-free canonical variables 1 and 3 (CV1 and CV3). AE, ethanol 100%; ET, ethanol 70%; CO, control; FO, formaldehyde 4%; FW, freshwater; MC, magnesium chloride; ME, menthol crystals; RE, refrigeration.

Table 2. *Pearson product-moment correlations between canonical variables (CV) and the residual value of each morphometric variable. Significant correlations (*P*<0.05) are in bold.*

Morphometric variables	CV1	CV2	CV3
Total length without branchial crown (TL)	**0.788**	**0.440**	0.107
Length of branchial crown (BC)	0.261	0.247	**0.381**
Length of thorax (TH)	**0.704**	–0.036	0.128
Length of setiger 1 (LS1)	0.315	–0.366	0.044
Depth of setiger 1 (DS1)	**–0.521**	0.121	0.144
Width of setiger 1 (WS1)	**–0.580**	**0.388**	–0.123
Length of setiger 4 (LS4)	**0.619**	–0.278	0.133
Depth of setiger 4 (DS4)	**–0.747**	0.176	–0.142
Width of setiger 4 (WS4)	**–0.683**	0.146	0.205
Length of setiger 8 (LS8)	**0.721**	0.071	–0.069
Depth of setiger 8 (DS8)	**–0.758**	–0.139	–0.239
Width of setiger 8 (WS8)	**–0.732**	0.085	0.059
Length of setiger 9 (LS9)	**0.645**	0.072	–0.014
Depth of setiger 9 (DS9)	**–0.815**	–0.247	–0.164
Width of setiger 9 (WS9)	**–0.658**	0.002	–0.074
Length of setiger 20 (LS20)	**0.470**	0.040	**0.378**
Depth of setiger 20 (DS20)	**–0.839**	0.136	–0.134
Width of setiger 20 (WS20)	**–0.620**	–0.129	**–0.370**
Length of setiger 50 (LS50)	**0.420**	0.068	–0.062
Depth of setiger 50 (DS50)	**–0.686**	**0.357**	–0.009
Width of setiger 50 (WS50)	**–0.530**	–0.122	0.110
Length of pygidium (LP)	–0.176	–0.234	–0.123
Width of pygidium (WP)	0.052	**–0.458**	–0.307

the other hand, specimens submitted to MC and ME with higher values of TL, TH, and LS (setigers 4, 8, 9, 20 and 50) and lower values of DS were more elongate, narrower, and flatter than living ones.

In relation to CV2, TL, WS1, and DS50 were positively correlated whereas width of pygidium (WP) showed a negative correlation. Specimens submitted to treatments RE, MC, and ME had higher values of WP, possessing wider pygidia than living specimens (CO). Other treatments apparently did not affect WP values when compared to CO.

Length of branchial crown (BC) and LS20 were positively correlated to CV3 whereas WS20 was negatively correlated. Specimens submitted to RE, FW, ET and MC procedures did not differ significantly from specimens submitted to control treatment (CO) as regards the length of branchial crown (BC), since all these specimens maintained the apex of the radioles curved (Figure 1). Nevertheless, specimens submitted to FO, AE and ME treatments presented higher values for BC than expected for CO, because these specimens stretched their radioles during the experimental procedure.

DISCUSSION

Our study provides information on how different anaesthetization and fixation methods may affect the shape of *Branchiomma luctuosum*, indicating how morphometric features were affected by each treatment. Experimental treatments clearly affected the shape of *B. luctuosum* as could be seen by the results of CDA and MANOVA and the percentage values of correct assignments.

Anaesthetization procedures

Depending on the study organism and which are the research goals, anaesthetization may be necessary. For example, when studying species that have an eversible proboscis, the anaesthetization procedure facilitates its eversion, which is extremely important for some morphological and taxonomic works. An isotonic magnesium chloride solution is commonly considered as the most useful anaesthetic in these cases (Fauchald, 1977; Amaral & Nonato, 1987; Rouse & Pleijel, 2001), since anisotonic solutions could damage specimens owing to morphological anomalies at cellular level (Dong et al., 2006).

However, some problems could arise with shape characters, especially when variation between species or within species is defined based on specimens submitted to several anaesthetization procedures, leading to misleading results. This serious problem has been observed already by other researchers (e.g. Howe, 2002; Jordaens et al., 2002). Anaesthetization with magnesium chloride (MC) or menthol crystals (ME) is commonly used to relax polychaetes, cnidarians, bryozoans, and aquatic molluscs (e.g. Lincoln & Sheals, 1979; Rouse & Pleijel, 2001). For *B. luctuosum*, MC and ME procedures resulted in specimens with more elongated, narrower and flatter bodies, and also in a wider pygidia than living specimens (CO). Body changes owing to the effect of MC were observed at the cellular level for some polychaetes (Pfannenstiel, 1982). Specimens submitted to refrigeration (RE), compared to CO, presented shorter, wider, and thicker bodies, but also presented wider pygidia like MC and ME specimens. Length changes caused by RE procedures have already been described for fish larvae (Fowler & Smith, 1983). For the branchial crown, only ME specimens were affected, stretching their radioles. Moreover, the integument was released from the body in ME and RE treated specimens. Consequently, with the integument also

the parapodial elements were lost (setae and uncini) which are essential characters for systematics (Fitzhugh, 1989). Most specimens submitted to MC also lost abdominal uncini.

Anaesthetization with freshwater (FW) is widely used for echinoderms, especially holothurians (Lincoln & Sheals, 1979), but there is no record of such treatment for polychaetes. *Branchiomma luctuosum* showed interesting results, as the specimens submitted to FW did not present significant alterations in measured features compared to the body shape of living specimens (CO). Therefore, the FW procedure could be successfully used in sabellids, particularly when dealing with purely shape description. However, the whole integument (including setae and uncini) was lost and the connection between the branchial crown and the thorax became very fragile. The integument of polychaetes is a simple and delicate structure, usually composed of a simple or pseudostratified layer of cells that rests on an extracellular matrix (Richards, 1978; Hausen, 2005). Polychaetes may also have a cuticle over the integument, which provides thickness and resistance for the body. However, many tubiculous polychaetes have secondarily lost the cuticle (Gardiner, 1992). The absence of a thick cuticle could explain the loss of the integument in *B. luctuosum* when submitted to ME and RE procedures. Moreover, the loss of setae and uncini hampers future taxonomic studies with the specimens, since they are essential taxonomic characters in sabellids (Fitzhugh, 1989).

Fixation without previous anaesthetization procedures

Fixation with 4% formaldehyde (FO) is widely applied, not only for polychaetes but also for many other invertebrate groups, such as cnidarians, sipunculans, crustaceans and chaetognaths (e.g. Lincoln & Sheals, 1979). For *B. luctuosum*, FO maintained the colour pattern, but resulted in a shorter, wider and higher body when compared to living specimens (CO). The branchial crown (BC) seemed to be longer than in CO specimens, due to the stretching of the radioles. Similar shape artefacts caused by FO procedure have been already reported for quite different organisms such as planktonic ciliates and fish (e.g. Dabrowski & Bardega, 1982; Choi & Stoecker, 1989; Sagnes, 1997). Besides shape deformations, there are some other disadvantages in using formaldehyde as fixative (Hopwood, 1996). The main disadvantages are that it is highly toxic, volatile, and is also a carcinogen, which requires many safety procedures (Roskams & Rodgers, 2002; Coggon et al., 2003).

Fixation with ethanol (AE and ET) is recommended not only as fixative but also as preservative for polychaetes and the major groups of invertebrates (Lincoln & Sheals, 1979). In almost all measured features, specimens submitted to EA and ET procedures maintained the values of CO, except for EA, which presented a higher value of BC. Similarly to specimens submitted to FO, this apparent increase took place due to the stretching of the radioles. Presumably, a similar effect was not observed in ET procedure because of the lower concentration of the fixative. In addition to preserving the living values of morphometric features and not being so toxic, the use of ethanol as fixative also allows the use of specimens for molecular techniques. Presently, molecular biology is considered an efficient approach to solve many systematic problems, and ethanol is considered to be an effective fixative for such purposes (Smith et al., 1987; Dessauer et al., 1996). Nevertheless, it must be remarked that the ethanol effects herein described, reflect a storage period of only ten days. Shrinkage by dehydration, a common effect of ethanol, could change shape in animals subjected to long term preservation as in museum material.

Morphology

Morphometric results obtained from *B. luctuosum* corroborate the initial idea that soft-bodied animals are differently affected by fixation procedures. Results from morphometric comparisons between populations or species that use different anaesthetization and/or fixation procedures should be used with care in order to prevent misleading conclusions. For example, when differences among populations are purely attributable to deformations caused by different fixation methodologies, care should be taken when interpreting inter-population variation as geographical variation.

For *B. luctuosum*, the best fixation methodologies to preserve the actual shape of the species are previous anaesthetization with freshwater (FW) or solely direct fixation with ethanol (AE and ET). Nevertheless, the choice of procedure should be based on the objectives of each study (Kruse & Dalley, 1990). Length of setiger 1 and length of pygidium appear to be valuable morphometric characters to potentially discriminate among populations, as they did not present deformation when submitted to different methodologies. However, even the total length of branchial crown (BC), which presented variation among methodologies, could also be very useful for taxonomic studies. Radioles of the branchial crown are generally composed of a columnar epithelium and cartilaginous skeletal cells (Perkins, 1984). Shape deformations are caused mainly by musculature action, and the absence of musculature in the radioles makes BC an important morphometric feature. The variation of BC values found in this work could be easily corrected if the radioles of all specimens had been stretched during measurement, since in RE, FW, ET, MC and CO the specimens maintained the apex of the radioles curved.

Species of the family Sabellidae and even the order Sabellida possess constrained morphology and habits (Orrhage, 1980; Smith, 1991; Rouse & Pleijel, 2001); for example all species are sedentary and live inside self-made tubes. Therefore, it is likely that the conclusions about the best anaesthetization/fixation procedures (FW, AE, and ET) and most promising characters (LS1, LP, and BC) for morphometric analyses could be applied not only for *B. luctuosum* species, but also for the whole family Sabellidae and other families of the order Sabellida.

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