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Shape matters: investigating the utility of geometric morphometric techniques in the deep-sea isopod family Macrostylidae (Isopoda: Asellota)

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Accurate taxonomic classification of deep-sea taxa is often impeded by the presence of highly morphologically similar but genetically distinct species. This issue is particularly pronounced in the isopods of the deep-sea family Macrostylidae, which exhibit remarkably low morphological variation despite significant genetic diversity. In this study, we present the first application of geometric morphometric techniques to 41 specimens across five species of deep-sea macrostylid isopods collected from Icelandic waters. Our results suggest that geometric morphometric techniques can effectively discriminate between macrostylid species. These techniques, hence, promise to be an important addition to the toolset of macrostylid taxonomists.

KEYWORDS

BIOICE, geometric morphometrics, integrative taxonomy, macrostylid, Iceland, IceAGE

1 Introduction

Geometric morphometrics has emerged as an important addition to the taxonomic toolset (Mutanen and Pretorius, 2007; Ludoški et al., 2008; Francuski et al., 2009; Roggero et al., 2013; Mitrovski-Bogdanović et al., 2014; Siriwt et al., 2015; Karanovic et al., 2016; Li et al., 2016; Grinang et al., 2019). This approach combines multivariate statistics with Cartesian coordinates to quantify shape variation, making it effective for identifying the subtle morphological differences that traditional taxonomic approaches may overlook (see e.g., Fukami et al., 2004; Bridge et al., 2023). Numerous studies have successfully applied geometric morphometric techniques to investigate cryptic species (usually defined as co-occurring species indistinguishable by the human eye despite high genetic distinctness), discovering new taxa, and identifying new taxonomically informative traits across a wide range of taxa (insects: Mutanen and Pretorius, 2007; Ludoški et al., 2008; Francuski et al., 2009; centipedes: Siriwt et al., 2015; copepods: Karanovic et al., 2016; decapods: Grinang et al., 2019; Lovrenčić et al.,

2020; Casaubon et al., 2023; ostracods: Ligios and Gliozzi, 2012; tardigrades: Fontoura and Morais, 2011). However, the application of geometric morphometrics is completely lacking for some taxonomic groups, including the deep-sea isopod family Macrostylidae Hansen, 1916.

The family Macrostylidae, comprising a single genus *Macrostylis* Sars, 1864, includes isopods with a global distribution spanning sublittoral (*M. spinifera* Sars, 1864, found at approximately 4 m) to hadal zones (*M. mariana* Mezhov, 1993, found at approximately 11,000 m) (Mezhov, 1992; Menzies, 1962; Brandt, 2002; 2004; Riehl et al., 2012). Despite an extensive distribution and significant molecular divergence, macrostylid isopods exhibit remarkably low morphologic variation (Riehl, 2014). Additionally, these isopods display varying degrees of sexual dimorphism, with copulatory (terminal) males having pronounced morphological differences from both subadult and females (Riehl et al., 2012), further complicating species diagnoses and allocation of conspecifics based solely on morphology.

Historically, macrostylid taxonomy has relied heavily on the comparative analysis of morphological characters and their linear measurements and ratios (Mezhov, 2003; Brandt, 2004; Vey and Brix, 2009; Riehl et al., 2012; Riehl and Brandt, 2013). Recent research on macrostylid isopods has increasingly employed integrative taxonomic techniques that combine molecular genetics and traditional morphometrics (Riehl et al., 2012; Riehl and Brandt, 2013; Bober et al., 2018). However, there are no known studies employing geometric morphometric techniques on Macrostylidae or other deep-sea isopods. This study represents the first application of geometric morphometric techniques in macrostylid taxonomy, aiming to: 1) evaluate the efficacy of these methods, and 2) to investigate various species of macrostylid isopods.

2 Materials and methods

Five species of macrostylid isopods (Figure 1) were used in this study: *M. spinifera* Sars, 1864, *M. sp. aff. spinifera*, *M. subinermis* Hansen, 1916, *M. longiremis* Hansen, 1916, and *M. magnifica* Wolff, 1962. The specimens used here (Table 1) were collected during research campaigns for several projects spanning from 1992 to 2014, including the BIOICE project (Benthic Invertebrates of Icelandic Waters; Brix and Svavarrson, 2010; Brattegard et al., 2019; Steingrímsson et al., 2020), the IceAGE project (Icelandic Marine Animals: Genetics and Ecology, 2008), and the PolySkag project (Polychaetes in coastal areas of the Skaggeyrak, 2014; Oug et al., 2015). All specimens analyzed here are deposited in the collections at the Senckenberg Natural History Museum in Frankfurt, Germany.

Initial species identifications were based on established taxonomic descriptions of macrostylid isopods (Sars, 1864, 1899; Meinert, 1890; Hansen 1916; Wolff, 1962). While there is some genetic data (16S and 18S, unpublished data) for macrostylids from the IceAGE and PolySkag projects, specimens collected during the BIOICE expedition lack genetic data due to the use of formaldehyde as a preservative.

A total of 41 subadult (preparatory) and adult (copulatory) specimens were used in geometric morphometric analyses. Only female isopods were used as they are more abundant in collections (Riehl et al., 2012) and are difficult to distinguish using morphology alone (Riehl et al., 2012), making them ideal candidates for use in geometric morphometric analyses.

The pleotelson was chosen as it is an important diagnostic character when used in conjunction with other morphological characters. It is also easier to position and photograph in a standard view compared to the third pereopod ischium or the operculum. As such, each pleotelson was photographed in dorsal view using a Leica M165C stereomicroscope equipped with a Leica DMC5400 20 Megapixel color CMOS camera. Images were saved in TIFF format using the Leica Application Suite (LAS X). The tpsUtil (Rohlf, 2015) program was used to prepare the images for landmarking and the tpsDig (Rohlf, 2015) program was used to digitize landmarks and semilandmarks. MorphoJ 1.07a (Klingenberg, 2011) was used for all subsequent analyses.

Though there are a few studies that have applied geometric morphometric techniques to isopod taxa (Santamaria et al., 2013; Ismail 2021; Kamilaris and Sfenthourakis, 2009; Kim et al., 2021), to our knowledge none have focused on macrostylid isopods. Previous studies on isopod taxa have focused primarily on body shape, on pleopodal appendages (Kamilaris and Sfenthourakis, 2009), or have employed destructive techniques (Bertin et al., 2002). Here, we selected homologous landmarks across macrostylid species that captured the most amount of shape variation using non-destructive techniques. Additionally, given the pleotelson is a symmetrical character (Schultz, 1969) and an excessive number of landmarks can reduce statistical power (Rufino et al., 2006; Mitteroecker et al., 2013), we selected three landmarks and 66 semi-landmarks comprising half of the pleotelson (Figure 2). Landmark 1 (lmk1) represents the point where the lateral pleotelson outline meets the 7th pereonite. Landmark 2 (lmk2) represents the midpoint of the posterior apex of the pleotelson, the position and shape of which appears to vary widely between macrostylids (e.g., *M. spinifera* vs. *M. subinermis*). Landmark 3 (lmk3) represents the maximum curvature of the point where the uropod inserts into the pleotelson (see arrow in Figure 2). The semi-landmarks are anchored between lmk 1 and lmk 2 and capture the lateral and posterior margins of the pleotelson.

A Procrustes superimposition method was used to standardize landmark data and generate Procrustes shape coordinates by translating, scaling, and rotating the raw coordinate data (Adams and Otárola-Castillo, 2013). The Procrustes shape coordinates generated were then used for principal component analyses (PCA) and canonical variate analyses (CVA). A PCA was performed to visualize and quantify pleotelson shape variation. The PCA created a morphospace (Figure 3) for visualizing shape variation, with each point representing the pleotelson shape of a macrostylid isopod. Points closer together on the PCA morphospace indicate more similar shapes, while points farther away indicate more dissimilar shapes. A CVA with a permutation test for pairwise distances (10,000



FIGURE 1 Female macrostyloid isopods used in the present study. (A) *Macrostyliis longiremis*; (B) *M. subinermis*; (C) *M. magnifica*; (D) *M. sp. aff. spinifera*; (E) *M. spinifera*.

TABLE 1 Macrostyloid specimens examined for the application of geometric morphometric techniques in this study.

| Expedition | Voucher | Species | Cruise ID | Station no. | Depth 1 (m) | Depth 2 (m) | Sampling Gear |
|-------------|-----------|----------------------|-----------|-------------|-------------|-------------|----------------------|
| BIOICE 2648 | BiMa 1218 | <i>M. longipes</i> | HM-1-94 | 14 | 1306 | 1310 | RP sledge |
| BIOICE 2648 | BiMa 1219 | <i>M. longipes</i> | HM-1-94 | 14 | 1306 | 1310 | RP sledge |
| BIOICE 2648 | BiMa 1220 | <i>M. longipes</i> | HM-1-94 | 14 | 1306 | 1310 | RP sledge |
| BIOICE 2648 | BiMa 1513 | <i>M. longipes</i> | HM-1-94 | 14 | 1306 | 1310 | RP sledge |
| BIOICE 2648 | BiMa 1514 | <i>M. longipes</i> | HM-1-94 | 14 | 1306 | 1310 | RP sledge |
| BIOICE 2648 | BiMa 1517 | <i>M. longipes</i> | HM-1-94 | 14 | 1306 | 1310 | RP sledge |
| BIOICE 2412 | BiMa 935 | <i>M. longipes</i> | B-9-93 | 562 | 1170 | 1174 | detr. sledge (Sneli) |
| BIOICE 2465 | BiMa 793 | <i>M. longiremis</i> | B-9-93 | 584 | 180 | – | detr. sledge (Sneli) |
| BIOICE 2465 | BiMa 794 | <i>M. longiremis</i> | B-9-93 | 584 | 180 | – | detr. sledge (Sneli) |
| BIOICE 2465 | BiMa 796 | <i>M. longiremis</i> | B-9-93 | 584 | 180 | – | detr. sledge (Sneli) |
| BIOICE 2465 | BiMa 798 | <i>M. longiremis</i> | B-9-93 | 584 | 180 | – | detr. sledge (Sneli) |
| BIOICE 2465 | BiMa 799 | <i>M. longiremis</i> | B-9-93 | 584 | 180 | – | detr. sledge (Sneli) |

(Continued)

TABLE 1 Continued

| Expedition | Voucher | Species | Cruise ID | Station no. | Depth 1 (m) | Depth 2 (m) | Sampling Gear |
|---------------|-----------|----------------------|-----------|-------------|-------------|-------------|---------------|
| BIOICE 2585 | BiMa 864 | <i>M. longiremis</i> | HM-1-93 | 43 | 450 | 450 | RP sledge |
| BIOICE 2585 | BiMa 872 | <i>M. longiremis</i> | HM-1-93 | 43 | 450 | 450 | RP sledge |
| BIOICE 2585 | BiMa 875 | <i>M. longiremis</i> | HM-1-93 | 43 | 450 | 450 | RP sledge |
| BIOICE 2863 | BiMa 01* | <i>M. magnifica</i> | B-8-96 | – | – | – | – |
| BIOICE 2863 | BiMa 02* | <i>M. magnifica</i> | B-8-96 | 734 | 2399 | 2399 | RP sledge |
| BIOICE 2863 | BiMa 04* | <i>M. magnifica</i> | B-8-96 | 734 | 2399 | 2399 | RP sledge |
| BIOICE 2863 | BiMa 06* | <i>M. magnifica</i> | B-8-96 | 734 | 2399 | 2399 | RP sledge |
| BIOICE 2863 | BiMa 07* | <i>M. magnifica</i> | B-8-96 | 734 | 2399 | 2399 | RP sledge |
| BIOICE 2863 | BiMa 09* | <i>M. magnifica</i> | B-8-96 | 734 | 2399 | 2399 | RP sledge |
| BIOICE 2904 | BiMa 904 | <i>M. n. sp. 1</i> | B-8-96 | 473 | 1057 | 1067 | RP sledge |
| BIOICE 2410 | BiMa 1436 | <i>M. n. sp. 1</i> | B-9-93 | 561 | 1074 | 1075 | RP sledge |
| BIOICE 2410 | BiMa 1439 | <i>M. n. sp. 1</i> | B-9-93 | 561 | 1074 | 1075 | RP sledge |
| BIOICE 2410 | BiMa 1440 | <i>M. n. sp. 1</i> | B-9-93 | 561 | 1074 | 1075 | RP sledge |
| BIOICE 2410 | BiMa 1441 | <i>M. n. sp. 1</i> | B-9-93 | 561 | 1074 | 1075 | RP sledge |
| BIOICE 2983 | BiMa 79 | <i>M. spinifera</i> | B-8-96 | 503 | 174 | 179 | RP sledge |
| BIOICE 2983 | BiMa 81 | <i>M. spinifera</i> | B-8-96 | 503 | 175 | 179 | RP sledge |
| BIOICE 2983 | BiMa 82 | <i>M. spinifera</i> | B-8-96 | 503 | 175 | 179 | RP sledge |
| BIOICE 2983 | BiMa 83 | <i>M. spinifera</i> | B-8-96 | 503 | 175 | 179 | RP sledge |
| BIOICE 2983 | BiMa 84 | <i>M. spinifera</i> | B-8-96 | 503 | 175 | 179 | RP sledge |
| BIOICE 2983 | BiMa 85 | <i>M. spinifera</i> | B-8-96 | 503 | 175 | 179 | RP sledge |
| BIOICE 2983 | BiMa 86 | <i>M. spinifera</i> | B-8-96 | 503 | 175 | 179 | RP sledge |
| BIOICE 2983 | BiMa 87 | <i>M. spinifera</i> | HM-1-93 | 43 | 450 | 450 | RP sledge |
| Polyskag 2014 | PMa 15 | <i>M. spinifera</i> | – | – | – | – | – |
| Polyskag 2014 | PMa 16 | <i>M. spinifera</i> | – | – | – | – | – |
| IceAGE | iMacro 21 | <i>M. longipes</i> | M85-3 | – | – | – | – |
| IceAGE | iMacro 37 | <i>M. longipes</i> | M85-3 | – | – | – | – |

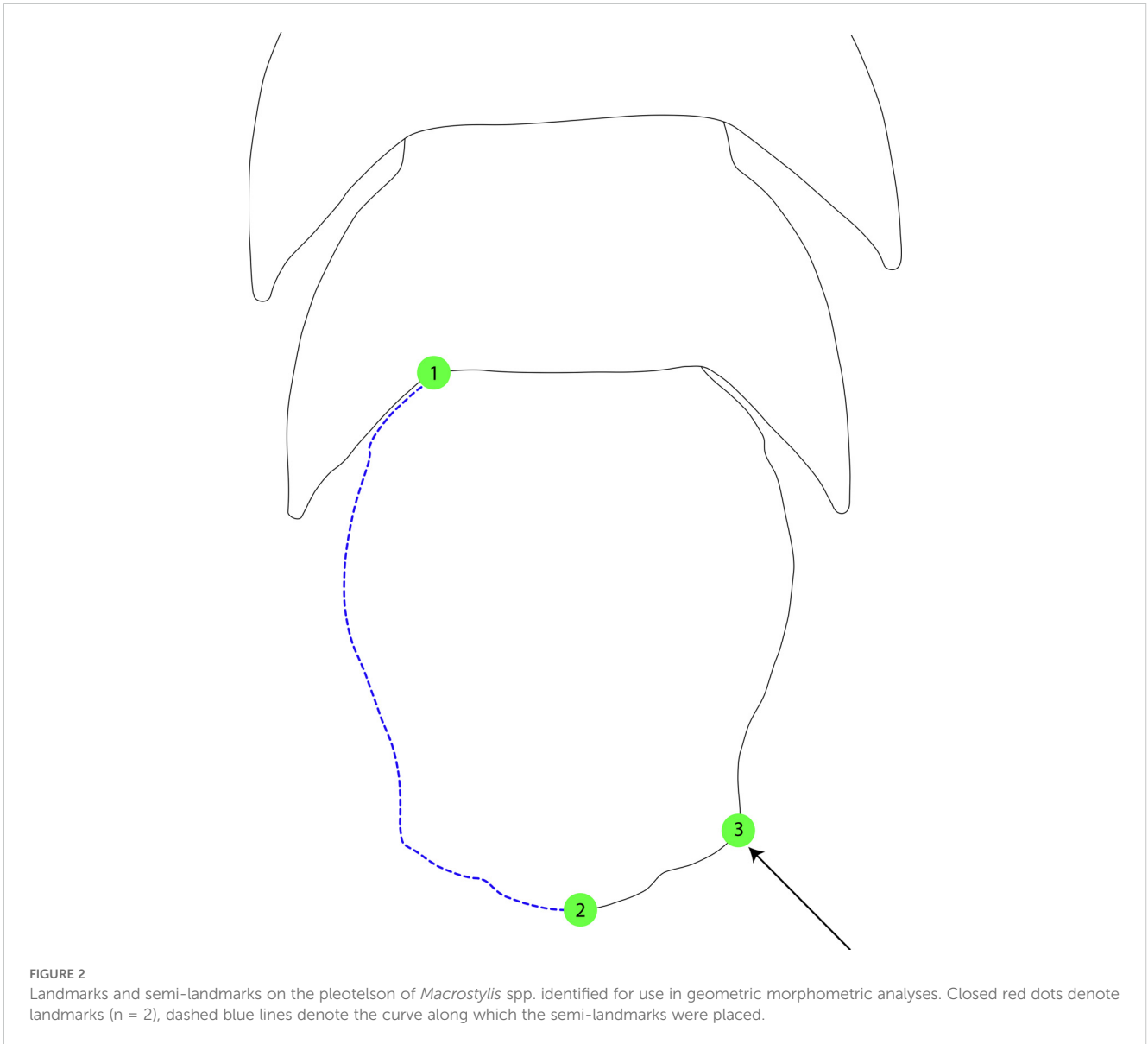
iterations) was performed to analyze interspecific shape variation in the pleotelson. The CVA (Figure 4) maximized the distance between individuals of different groups (i.e., species) while minimizing the distance between individuals of the same groups.

The Procrustes and Mahalanobis distances (Table 2) generated from the CVA were used to determine the statistical significance of the permutation tests. The Procrustes distance measured the absolute magnitude of shape deviation (Klingenberg and Monteiro, 2005), while the Mahalanobis distance measured how different an individual species was from others (Klingenberg and Monteiro, 2005).

Finally, a Procrustes ANOVA (analysis of variance; pANOVA) was conducted to assess the significance of pleotelson shape variation between the macrostyliids studied here. The significance level (p-value) for all analyses was set to 0.05, with p-values less than 0.05 considered statistically significant.

3 Results

The results of the pANOVA revealed significant differences in pleotelson shape between macrostyliid species ($p < 0.0001$; Table 2). The first two principal components accounted for 80.9% (PC1 53.3%, PC2 27.6%) of total variance. A scatter plot of the first two principal components (Figure 3) showed *M. spinifera* and *M. sp. aff. spinifera* clustering together but not overlapping in their distribution in the negative PC axis, *M. subinermis* forming its own distinct cluster in the positive PC axis, and *M. magnifica* slightly overlapping with *M. longiremis* in the positive PC axis. The first principal component accounted for most of the morphological variation and primarily showed changes in the posterolateral margins and the positioning of the pleotelson posterior apex. The pleotelson shape of species on the negative PC1, i.e., *M. spinifera*



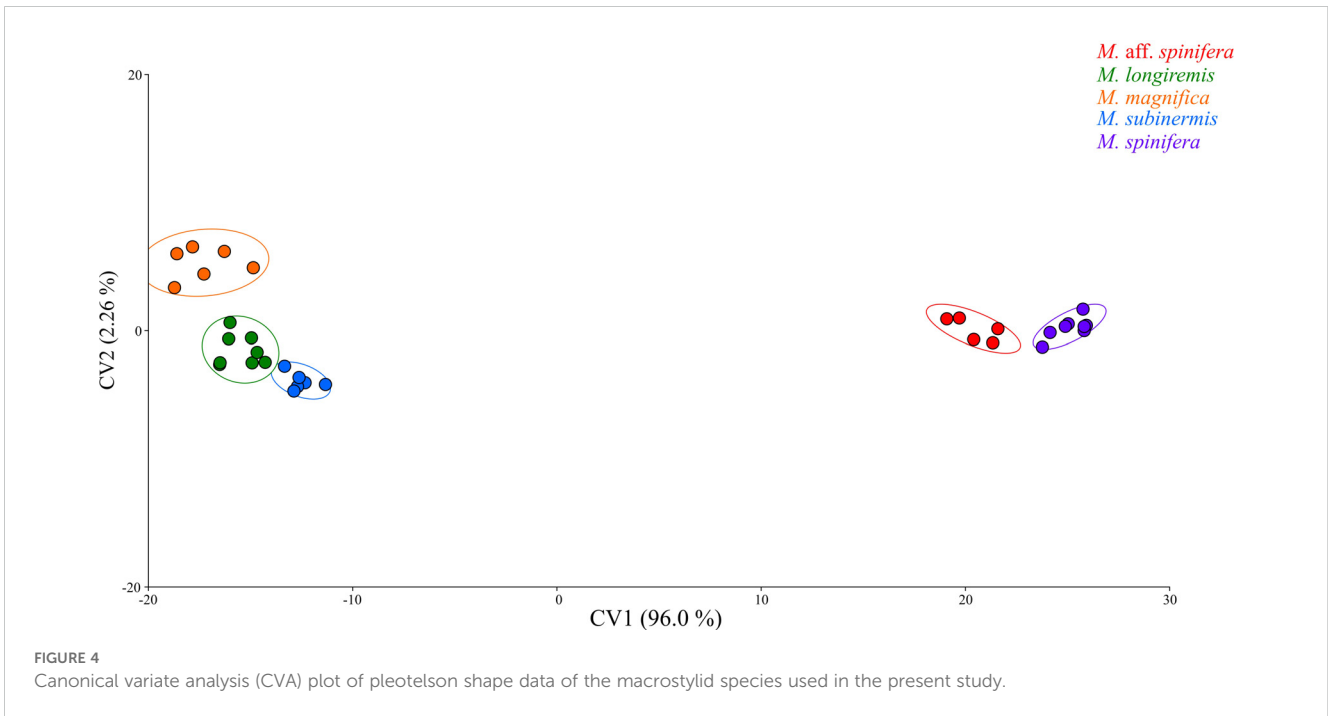
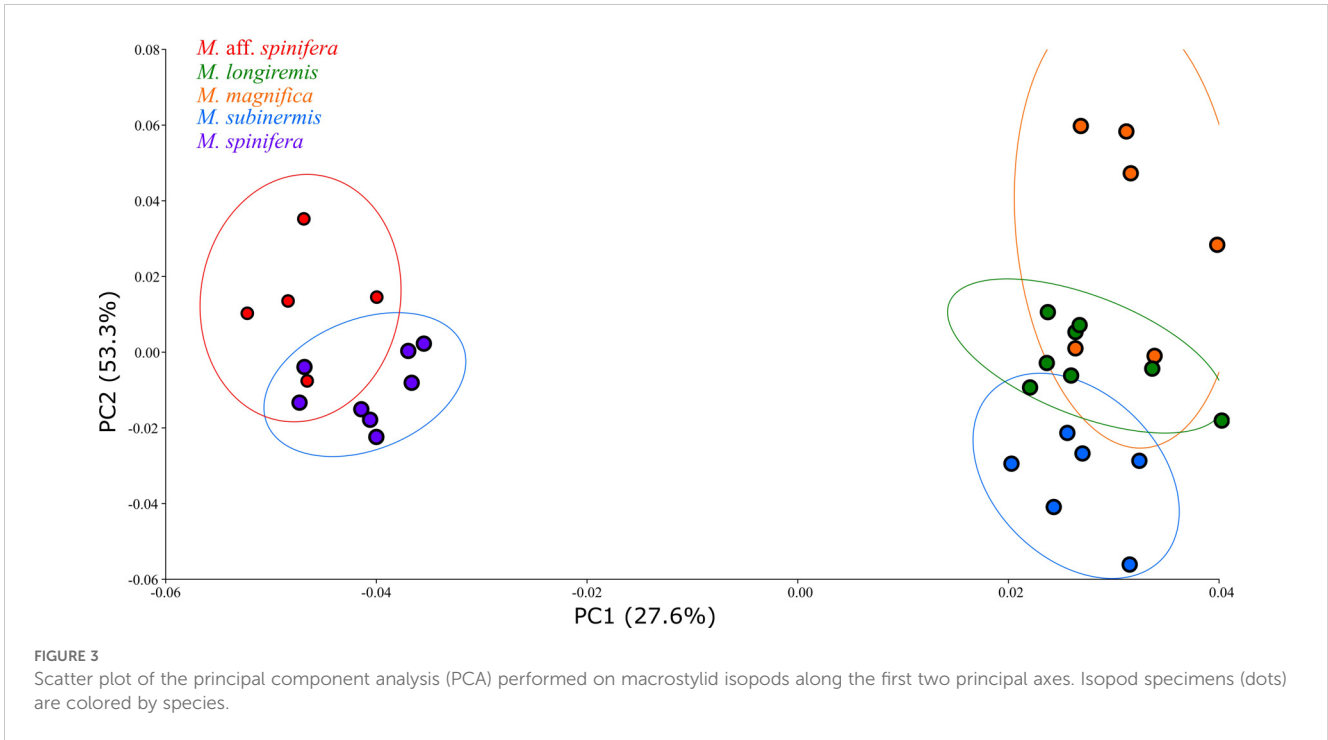
and *M. sp. aff. spinifera*, was more hourglass shaped with a waist. The posterolateral margins and the posterior apex of these two species are very different from the remaining species clustered on the positive PC2 axis. The second principal component primarily showed changes in the lateral margins and overall pleotelson shape.

The first two canonical variates accounted for 98.3% (CV1 96.0%, CV2 2.26%) of total variance. A scatter plot of the first two canonical variates (Figure 4) showed all five species completely separated. *Macrostylis spinifera* and *M. sp. aff. spinifera* clustered closely together but did not overlap in the positive CV1 axis, *M. subinermis* and *M. longiremis* overlapped in their distribution in the negative CV1 axis while *M. magnifica* clustered by itself in the extreme negative CV1 axis. On average, the species on the positive extremes, i.e., *M. spinifera* and *M. sp. aff. spinifera*, were characterized by an hourglass shaped pleotelson with a waist while the species on the negative extremes, i.e., *M. magnifica*, *M. subinermis*, and *M. longiremis*, were characterized by a pleotelson

with more parallel lateral margins. Similarly, the second canonical variate (CV2) axis also demonstrated shape changes in the posterior end of the pleotelson, with the species on the positive extremes having a less pronounced waist, compared to the isopods on the negative extremes having a more pronounced waist.

4 Discussion

Our application of geometric morphometric techniques to the pleotelson of macrostyloid isopods successfully differentiated between the five species studied here, revealing subtle morphological differences even between highly morphologically similar taxa. Despite their high levels of genetic divergence, macrostyloid isopods exhibit remarkable morphological homogeneity (Riehl and Brandt, 2010; Riehl and Brandt, 2013) significantly impeding accurate species diagnoses and complicating efforts at taxonomic reorganization. Our



results demonstrate that geometric morphometric techniques are excellent at detecting the subtle morphological differences that separate species which are highly morphologically similar.

As a prime example, the lack of overlap between *M. spinifera* and *M. sp. aff. spinifera* was unexpected given the high morphological similarity their females exhibit. Instead, geometric morphometric techniques clearly discriminated the two species. The distinction between *M. spinifera* and *M. sp. aff. spinifera* is ostensible in their males. The males of *M. sp. aff. spinifera* have strikingly elongated sixth

TABLE 2 Procrustes ANOVA for the pleotelson shape of all species used in this study.

| Effect | SS | MS | df | F | p |
|------------|------------|--------------|------|---------|----------|
| Individual | 0.05985144 | 0.0001133550 | 528 | 9.23.55 | < 0.0001 |
| Residual | 0.01778706 | 0.0000048125 | 3696 | - | - |

SS, sum of squares; MS, mean squares (i.e., SS divided by df); df, degree of freedom.

and seventh pereopods that closely resemble those of *M. longipes*. However, this rather strong expression of sexual dimorphism impedes allocation of conspecific male and female specimens (see also, e.g., Riehl et al., 2012); without the application of geometric morphometrics or genetics, the females may easily be taken for *M. spinifera* by an unexperienced identifier while the males may be identified either as *M. longipes*, or as a separate species without conspecific females in the samples. Interestingly, Hansen (1916) was the first one to report variation within *M. spinifera*. He remarked on an “atypical form” of a female macrostyloid collected from the Davis Strait, which differed from the “typical form” of *M. spinifera* in its pleotelson shape along with differences in other morphological characters. More recently, a Bayesian phylogenetic reconstruction using 16S mitochondrial DNA from macrostyloids recovered *M. spinifera* in two clades (Riehl, 2014). Hansen’s (1916) remarks on a different form of *M. spinifera*, coupled with phylogenetic results (Riehl 2014, unpublished chapter¹) suggest that there may be more than one species hidden under *M. spinifera*. As shown here, an approach integrating geometric morphometrics is useful for further investigating the morphological differences between females of these two species.

While our results demonstrated a successful application of geometric morphometric techniques, our study was limited in several respects. First, we were restricted by a small sample size ($N = 5-10$) for all species excluding *M. spinifera*. A small sample size is correlated with an increased risk of type II error and reduces the power of the statistical analyses being undertaken (Columb and Atkinson, 2016). Second, because of a limited number of specimens available to us and the enormous and cost-prohibitive sampling efforts required to collect deep-sea specimens, we avoided dissections or any techniques that would physically damage specimens. As such, this study was limited to only the pleotelson as it was the easiest to standardize without dissection. Still our results demonstrate the potential of geometric morphometrics as a powerful tool in macrostyloid taxonomy, highlighting efficiency and applicability without the necessity of time-consuming and specimen-harming preparation.

Our findings introduce a promising new direction for research in macrostyloid taxonomy and suggest that geometric morphometric techniques are a useful addition to the existing set of tools used in this field. As shown here, geometric morphometric techniques are especially useful for elucidating shape differences between female macrostyloid isopods, which can be difficult to differentiate using traditional morphology. Within the scope of macrostyloid taxonomy, we expect that future studies will integrate these techniques in their approach and expand the use of geometric morphometrics to not only assess other diagnostically informative body parts, such as the fossosome and the operculum but also assess the efficacy of new taxonomic characters in macrostyloid taxonomy. Outside the scope of macrostyloid taxonomy, geometric morphometric techniques may be applicable to other deep-sea isopod families to support taxonomic efforts. Geometric morphometric techniques may also be useful in other deep-sea asellotes that face similar difficulties in efforts at taxonomic reorganization (Raupach et al., 2009; Brix et al., 2011).

Data availability statement

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding author.

Ethics statement

The manuscript presents research on animals that do not require ethical approval for their study.

Author contributions

AC: Data curation, Formal analysis, Investigation, Methodology, Validation, Visualization, Writing – original draft, Writing – review & editing. TR: Data curation, Funding acquisition, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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