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RESEARCH ARTICLE **Open access**

Identifying closely related species of the genus *Gammarus* (Crustacea, Amphipoda) using geometric morphometrics

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Abstract
Landmark-Based Geometric Morphometric Methods were used for the first time to quantitatively assess shape variations of the third epimeral plate (Ep3) of six *Gammarus* species (*G. lordeganensis, G. parthicus, G. pretzmanni, G. pseudosyriacus, Gammarus* sp1 and *Gammarus* sp2.) from five different localities in Iran. Two landmarks and 10 semi-landmarks on the posterior, anterior and inferior margins of the Ep3 were digitized on 78 adult male specimens. Shape diversity of samples and discrimination of all species were analyzed with Principal Components Analysis (PCA) and Canonical Variates Analysis (CVA). The results strongly supported the distinction in the posterior margin of Ep3 shape of the six species, while the inferior margin clearly showed similar morphological structure. A remarkable separation of *G. lordeganensis* as a distinct group from the rest of the species was found in both CVA and PCA analyses of anterior margin of Ep3 shape, while other species had overlaps with each other. Based on these findings, geometric morphometric data, could be used to identify diagnostic morphological traits. The shape of the Ep3 could be used as an appropriate character for separating closely related amphipod species of the genus *Gammarus*.

Key words: *Gammarus*, third epimeral plate, geometric morphometrics, shape variation

INTRODUCTION

Geometric morphometrics is an efficient technique to analyze the variability of the biological structures and specify the exact nature and position of the morphological shape differences since the late 1980s (Hennessy & Stringer, 2002; Rosas & Bastir, 2002). Landmark-based geometric morphometrics captures shape information and geometry of the object effectively to study population variations through a powerful and comprehensive statistical analysis (Rohlf, 1999; Cadrin, 2000). Also, semi-landmarks describe information on curves and outlines in this method (Gunz & Mitteroecker, 2013). Consequently, Geometric morphometrics provides not only large amounts of significant shape information that previously was unattainable through traditional morphometric approaches, but also is cost-effective, quick, and accurate. (Zeltdich *et al*. 2004, 2012; Grinang *et al.* 2019).

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Crustaceans are a suitable group for using geometric morphometrics technique due to their tough integument and easily identifiable homologous landmarks (Rosenberg, 2002; Rufino *et al*. 2004; Riedlecker *et al.* 2009; Hampton *et al*. 2014). In this group, amphipods are a good model for the application of this methodology and the geometric study of the anatomical shapes (Curatolo *et al*. 2013). Among the various amphipod genera, *Gammarus* Fabricius, 1775 has the highest diversity and till now 18 valid species of genus *Gammarus* have been identified from the different freshwater regions of Iran (Zamanpoor *et al*. 2011). The Ep3 as a specific morphological characteristic of the genus *Gammarus* was selected for this analysis because this anatomical piece has various forms and consistent variations among different species, and it optimizes the taking of photographs and geometric morphometric analysis (Curatolo *et al.* 2013).

There are no studies to apply the geometric morphometrics technique as a powerful tool for examining the Ep3 shape variation and discrimination of different *Gammarus* species. So, this study aims to analyze the Ep3 shape variation as a diagnostic character using a geometric morphometric method for the first time in the genus *Gammarus* to discriminate different *Gammarus* species.

MATERIALA AND METHODS

The samples were collected from different five locations in Chaharmahal-Va-Bakhteyari, Markazi, and Fars provinces between 2016 and 2017. Characteristics of sampling areas are presented in Table 1 and figure 1. In the laboratory, the specimens were classified according to sex and maturity, and only mature and male *Gammarus* species were used in this study. Several keys to the *Gammarus* were used to identify these species (Karaman & Pinkster, 1977; Stock *et al*. 1998; Khalaji-Pirbalouty & Sari 2004, 2006).

Thirteen adult males *Gammarus* specimens from each populations were used for geometric morphometrics analysis. We examined a total of 78 Ep3s from all five locations in this study. Digital images of the Ep3s were captured using ZEISS Axiocam ERc 5s camera, mounted on a Carl ZEISS microscope. Anterior, posterior and inferior margins of Ep3s were separately analyzed through landmark-based morphometric methods. The digital images were processed with MakeFan6 software. MakeFan6 software was used to draw parallel lines at equal distances along a curve for placing semi-landmarks on digital images (Sheets, 2003; Curatolo *et al.* 2013).

Then the coordinates of two landmarks and 10 semi-landmarks were digitized separately on each of the anterior, posterior, and inferior margins of Ep3s digital images (Fig. 2A-C) using TpsDig2 v. 1.11 software (Rohlf, 2004; Curatolo *et al*. 2013). Subsequent analyses of the landmark data were conducted using Paleontological Statistics (PAST) software (Hammer *et al.* 2001). A generalized Procrustes analysis (GPA) was applied to remove the variations not related to the form such as position, orientation, rotation, and scale (Rohlf, 1999, Zelditch *et al*. 2004). Shape variation of samples was analyzed with Principal Components Analysis (PCA) followed by Canonical Variate Analysis (CVA) using the PAST v. 3.04 software for simplifying the description of differences between groups (Hammer *et al*. 2001). Also, PCA was performed to determine the variation explained by each principal component (PC). The total variability by principal component axis and canonical variate axis were included in figs. 3-5.

TABLE 1. Sampling locations of *Gammarus* species.

No	Locality	Coordinates	species
1	Charmahal va bakhteyari, Borujen, Bizhgerd spring	31°45'56.63"N, 1°10'19.76"E	*Gammarus* sp1.
2	Charmahal va bakhteyari, Farsan , Pir-Ghar spring	32°13'1.32"N, °32'38.66"E	*Gammarus* sp2.
3	Fars, Eghlid, Rasoul spring	30°53'27.61"N 52°40'18.31"E	*G. pseudosyriacus*
4	Charmahal va bakhteyari, lordegan, Barm spring	31°30'32.57"N 50°49'28.68"E	*G. lordeganensis*
5	Markazi, Shazand, Sarab abbas abad spring	33°54'37.78"N 49°25'29.82"E	*G. pretzmanni,* *G. parthicus*

RESULTS

The Geometric morphometrics analyses were conducted to obtain Ep3 shape variations between different species of genus *Gammarus*. The digital images of 78 third epimeral plates were compared using PCA and CVA.

The PCA performed on 13 Ep3 posterior margin shape coordinates of two landmarks and 10 semi-landmark from the six studied species resulted in 24 principal components, with the first two components explaining 94.78 % of the total variation (PC1 82.30 %, PC2 12.48 %) (Fig 3). To get a clearer picture of the segregation of species, CVA performed on the same dataset revealed that

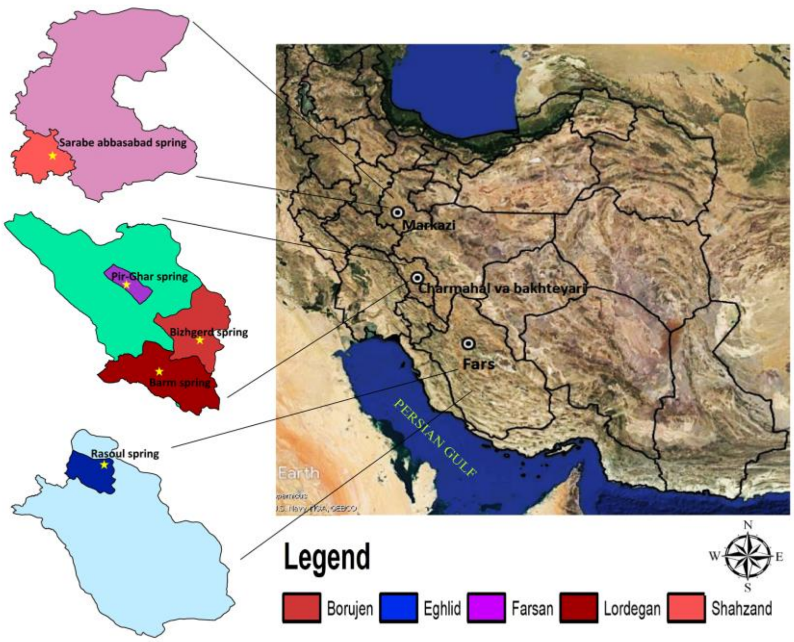

FIGURE 1. Map showing surveyed areas in Iran between 2016 and 2017.

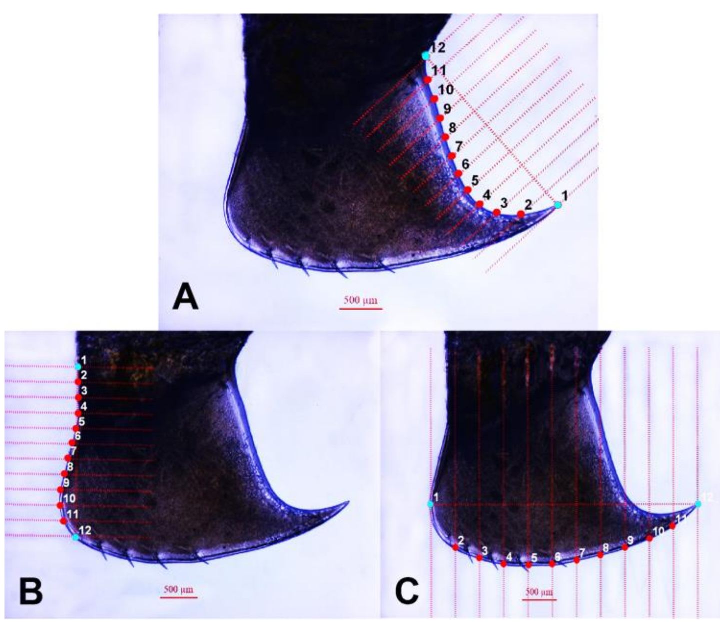

FIGURE 2. Position of the landmarks and semi-landmarks on the third epimeral plate for geometric morphometric analysis. The landmarks (points 1 and 12) and the semi-landmarks (points 2 to 11). (A) Posterior margin of third epimeral plate; (B) anterior margin of Ep3; (C) inferior margin of Ep3.

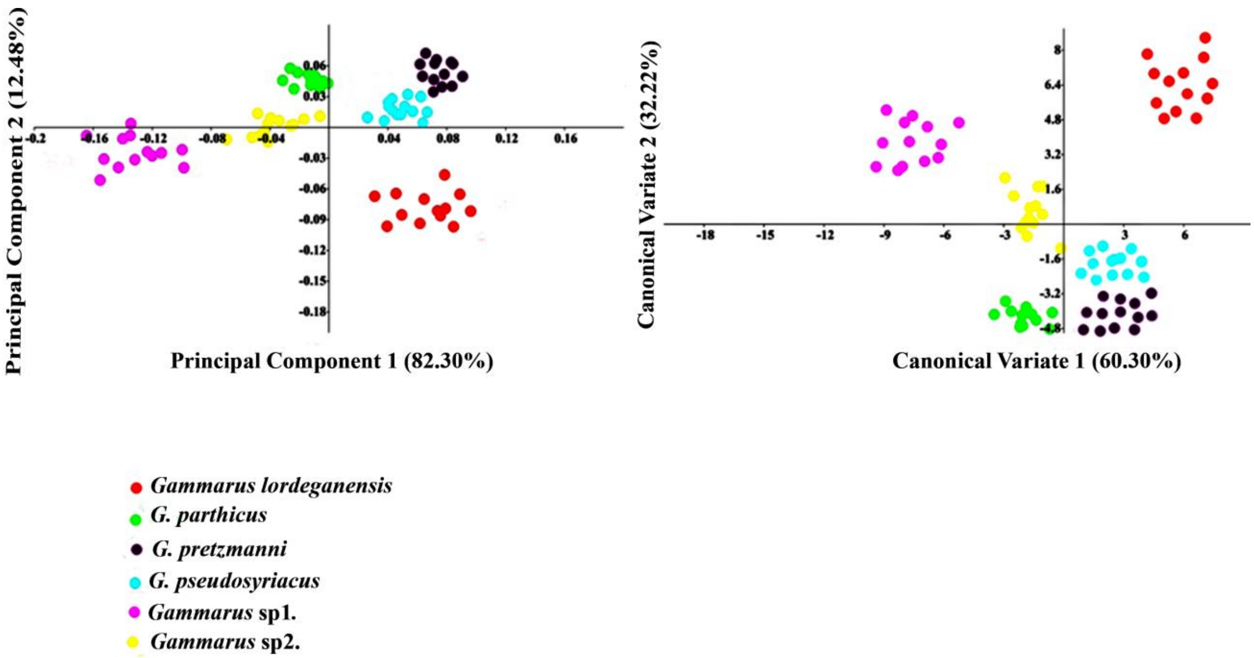

FIGURE 3. PCA and CVA showing significant difference in the shape of the posterior margin of Ep3 between different species of genus *Gammarus.*

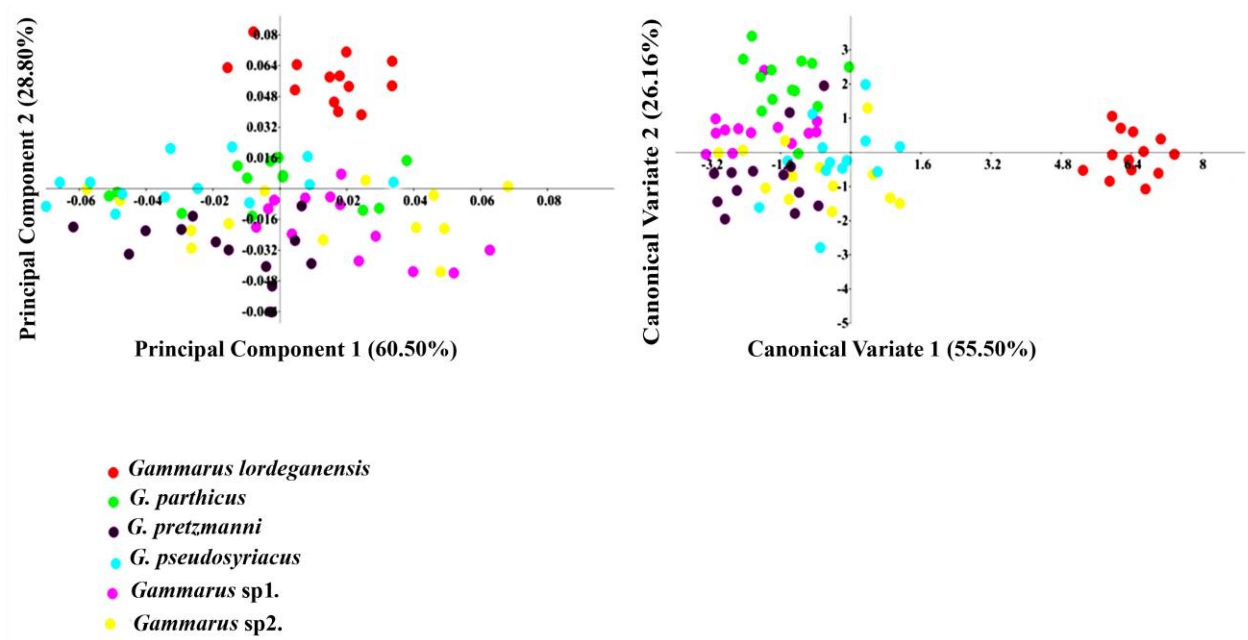

FIGURE 4. PCA and CVA showing insignificant variations in the shape of the anterior margin of Ep3 between different species of genus *Gammarus* except *G. lordeganensis* that is clearly distincted from the rest species.

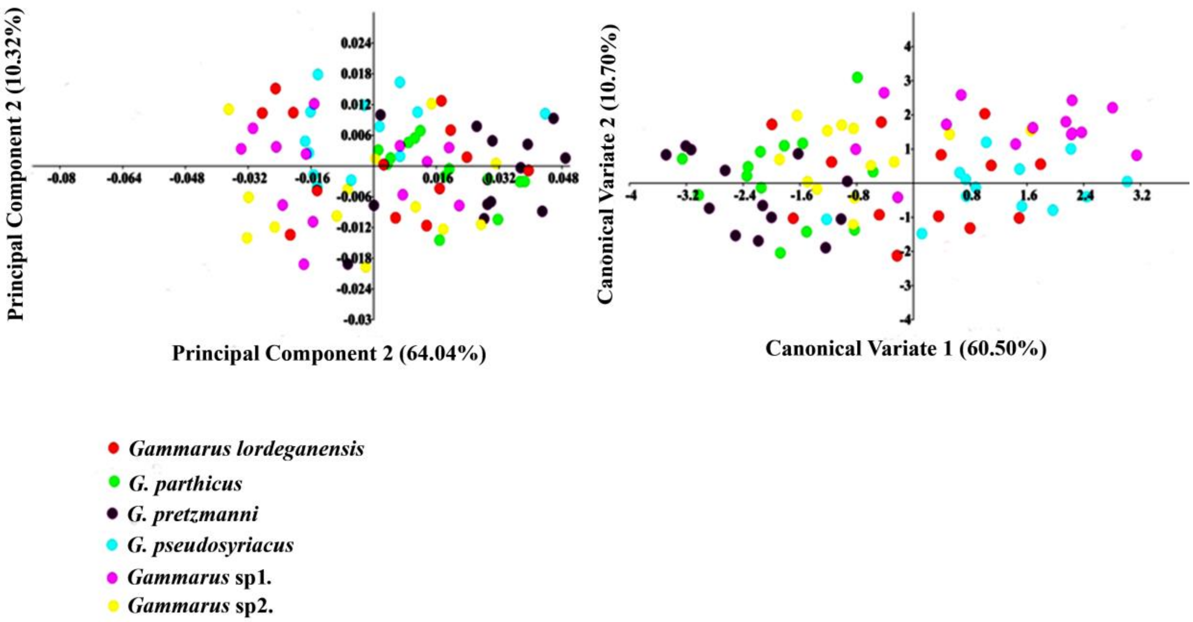

FIGURE 5. PCA and CVA showing insignificant variations in the shape of the inferior margin of Ep3 between different species of genus *Gammarus*.

the first two Canonical Variates (CV1 60.30 %, CV2 32.22 %) explained 92.52 % of the total variation (Fig. 3). So, significant morphometric differentiation in the posterior margin of Ep3 shape between different species of genus *Gammarus* were evident. These populations were completely separate and no overlap occurred among them. Over 88% of the anterior margin of Ep3 shape variation was described by principal components 1 and 2. PC1 and PC2 represent 60.5% and 28.8% of the variation, respectively (Fig. 4). This result is confirmed by CVA. The two first Canonical Variates (CV1 and CV2) explained 81.66 % of the total variation. The CV1 and CV2 explained 55.5% and 26.16% of the total variation between the groups, respectively (Fig 4). Results of the multivariate analyses clearly show that variation in the anterior margin of Ep3 shape is insignificant ($P>0.05$). There is only a significant difference between *G. lordeganensis* with other groups ($P<0.05$). A remarkable separation of *G. lordeganensis* as a distinct group from the rest of the species was found in both CVA and PCA analyses. Other species have overlap with each other.

PC1 and PC2 depict 64.04% and 10.32% of the variance in the shape of the Ep3 inferior margin between the groups, respectively. Results of PCA are supported by CVA. The first canonical axis and the second canonical axis explained 60.5% and 10.7% of the total variation between the groups, respectively (Fig. 5). Multivariate analyses clearly show that variation in the inferior margin of Ep3 shape is insignificant ($P>0.05$). All specimens were widely overlapped in the scatter plots.

DISCUSSION

This investigation is the first study to utilize the geometric morphometric technique to quantify Ep3s morphological variation for differentiation of *Gammarus* species. In our study, landmark-based geometric morphometric technique has been used to analyze differences in the posterior, anterior, and inferior margins of Ep3 shape of six *Gammarus* species and species discrimination. The results of the posterior margin of Ep3 shape analyses clearly showed that all species were completely distinct and different from each other. The posterior margins of the Ep3 shape of each species have different and various forms. The posterior margin of the Ep3 of *Gammarus* sp1 is very acuminate. The form of the Ep3 of the *G. lordeganensis* is convex posteromedially, while the shape of Ep3 of *G. parthicus* is weakly pointed posteroventrally. Ep3 shape of the *G. pretzmanni* has a rectangular posteroventral corner. The posterodistal corner of *G. pseudosyriacus* Ep3 is slightly pointed and the Ep3 posterodistal corner of *Gammarus* sp2. is sharply pointed.

These results confirm previous studies on the Ep3 posterior margin shape of the genus *Bathyporeia* (Amphipoda, Bathyporeiidae). The Ep3 shape especially the postero-ventral tooth of the Ep3 was used as a diagnostic character to identify *Bathyporeia* species (Bellan-Santini, 1989; d'Udekem d'Acoz & Vader, 2005). The geometric morphometrics technique has also been conducted on the Ep3s of the Mediterranean *Bathyporeia guilliamsoniana* (Spence Bate, 1857) to assess intra-specific variations (Curatolo *et al*. 2013). It was found that *B. sunnivae* and *B. megalops* are morphotypes with *B. guilliamsoniana* and that the geometric morphometrics method helps to identify and discriminate species (Curatolo *et al*. 2013).

A considerable separation of *G. lordeganensis* as a distinct group from the rest of the species was observed in both CVA and PCA analyses of Ep3 anterior margin shape. The differentiation of *G. lordeganensis* from the other species is due to the different form of the anterior margin of Ep3 shape which is lobbed antero-inferiorly. Other species overlapped with each other due to having similar forms of the anterior margins of Ep3s which are not lobbed antero-inferiorly. Geometric morphometrics analysis of the inferior margin of Ep3 clearly shows that all specimens were entirely overlapped in the scatter plots due to their same morphological structure. These findings revealed that the shape of the third epimeral plate, particularly its posterior margin, is a better characteristic to diagnose species in the genus *Gammarus* (Fig 6).

This technique was used successfully in amphipods by Riedlecker *et al* (2009) on the second gnathopod propodus to discriminate native and non-native species of Caprellidae. Moreover, the geometric morphometric technique was effectively performed on species of the genus *Bathyporeiidae*, to assess the 'cryptic' variation in the Ep3 shape and species identification (Curatolo *et al*. 2013). In addition, this method was used in another group of amphipods by Layeghi *et al* (2019) for investigating of sexual dimorphism of taxonomic characters. In their study, sexual dimorphism of gnathopods 1 and 2 and uropod 3' shapes in *Parhyale darvishi* was successfully described using geometric morphometric method (Layeghi *et al.* 2019). Application of geometric morphometrics in the present study proved to be a very valuable tool for shape analysis, separating species and studying morphological variation in this group of amphipods. Ep3 shape makes amphipods, especially those of the genus *Gammarus* good models for identifying species using the geometric morphometrics method.

Several previous studies have confirmed the applicability of the geometric morphometric technique in crustaceans to identify species and morphological discrimination of various species, differences of anatomical shapes, revealing sexual dimorphism, shape and size variation, and identifying taxonomic key characteristics of species (Giri & Collins, 2004; Zimmermann *et al*. 2012; Ligios & Gliozzi, 2012; Bissaro *et al.* 2013; Marchiori *et al*. 2014; Diawol *et al*. 2015; Karanovic *et al*. 2016; Grinang *et al.* 2019). Research by Bagheri et al (2020) confirmed that geometric morphometric technique combined with statistical methods on the variation of carapace shape of blue swimming crab *Portunus segnis* (Forskal, 1775) along the Iranian coasts of the Persian Gulf and Oman Sea are useful in separating *Portunus* species (Bagheri *et al*. 2020). Recent studies on pattern of shape variation in isopods have received much attention. A landmark geometric morphometric approach was applied on dactylus shape of cymothoid isopods to investigate size algometry and shape variations. The results revealed that geometric morphometric method is an effective tool for uncovering complex patterns from simple outline shapes like dactyli (Baillie *et al.* 2019). Geometric morphometric method was able to show the seasonal shape variations in terrestrial isopod *Porcellionides pruinosus* as well as shape sexual dimorphism (Ismail, 2021). 2D landmark-based geometric morphometrics was effectively applied to study of interpopulation size of a Microcerberid Isopod and pleon sensilla, and male pleopod II endopodite shape variations. It was

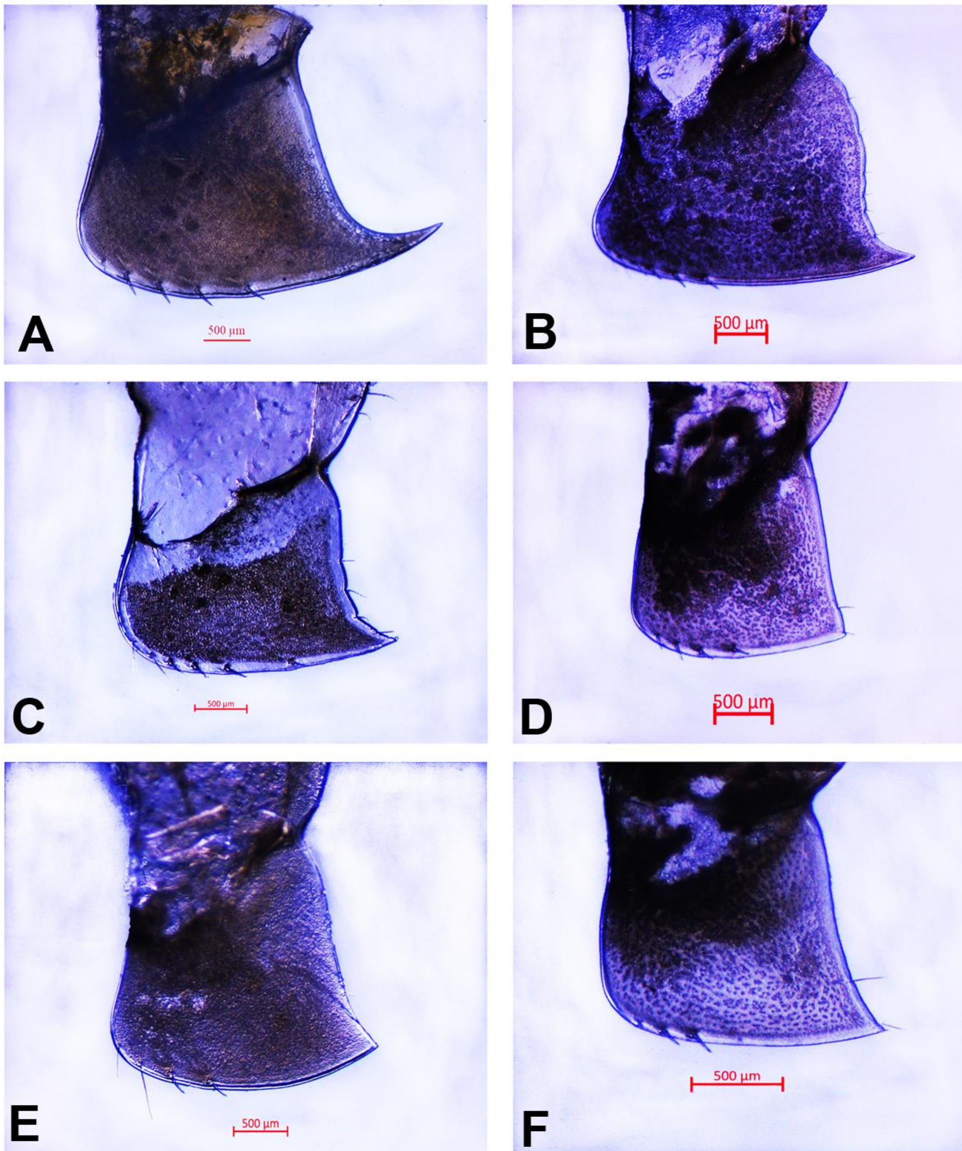

FIGURE 6. The third epimeral plates of different species of genus *Gammarus*. A. *Gammarus* sp1. B. *G. lordeganensis*, C. *Gammarus* sp2. D. *G. pretzmanni*, E. *G. pseudosyriacus*, F. *G. parthicus*

found that this method as a novel approach provides new insight to intrapopulation shape variations and ontogenetic variation (Kim *et al.* 2021).

All of the above studies in different groups of crustaceans as well as this research confirmed that 2D landmark-based geometric morphometric method is able to show shape variations and species discrimination

CONCLUSION

This study represents the first attempt to utilize the geometric morphometrics method for morphological analysis of the third epimeral plates in the genus *Gammarus*. Based on our geometric morphometrics analyses, Ep3 shape particularly its posterior margin across the species can be the best characteristic for species discrimination in the genus *Gammarus*. Thus, the geometric morphometrics method can be a precise tool for shape analysis of *Gammarus* species. Moreover, Amphipods especially those of the genus *Gammarus* are appropriate for the geometric study of the anatomical shapes.

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