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Novel validating indices to indicate sexual differences in the horsehair crab *Erimacrus isenbeckii* (Brandt, 1848)

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Abstract

Crustaceans exhibit a great diversity of morphological characteristics that are useful for inferring the general habits of species. In cheiragonid brachyuran crabs such as the horsehair crab *Erimacrus isenbeckii*, which live in the cold water, less attention has been paid to differences in external morphology between sexes. Our group recently identified several morphological characteristics on the carapace of the horsehair crab to distinguish females from males by geometric and measurement-based morphometric approaches. In this study, we constructed 3D images of the carapace that were then applied in the establishment of new validation indices to identify adult females and males. At first, the density patterns of tubercles with setae on the carapace were different between sexes. Even in sexually-matured individuals, smaller females had more dense tubercles on the carapace than males. Second, the curvature radius of the cross-section of the carapace also showed clear differences between sexes. The carapace of females was rounder than that of males. This study provides new tools to distinguish females from males using 3D imaging, and is the first evidence to quantitatively show sexual differences in “roundness” of the carapace in the horsehair crab. 3D-based morphometrics using curvature radius will be a useful index that can be applied for geographic comparisons and for other brachyuran species.

Key words: *Erimacrus isenbeckii*; 3D scanning; carapace; protrusion; seta; sexual differences

Introduction

The cheiragonid brachyurans are generally known as cold water crabs, and three species (the horsehair crab *Erimacrus isenbeckii*, and the helmet crabs *Telmessus acutidens* and *T. cheiragonus*) are important representative fishery crabs in Japan (Yanagimoto 2007). They are widely distributed in the North Pacific from the Alaskan coast via Kamchatka to the Northern part of Japan, mainly in the Hokkaido area (Sasaki 2003). In particular, the horsehair crab is Hokkaido’s most important commercial resource. To date, no attention has been paid to sexual differences in the morphology in *E. isenbeckii*, although several studies focusing on embryogenesis (Nagao et al. 1999), larval development (Ichikawa et al. 2013, 2014a, 2014b, 2018; Jinbo et al. 2005, 2007), sex pheromones (Asai et al. 2000; Dixon et al. 2005), and genetic variation in Japan (Azuma et al. 2008) have been conducted. Brachyuran crabs show conspicuous sexual dimorphism in the shape of abdomen, pleopod structure and position of gonopore. However, alternative morphological index may be necessary for specimens such as fossils or stomach content having lost these sex-specific characteristics. To breakthrough this limitation, our group recently demonstrated several morphological characteristics on the carapace to distinguish females from males using morphometric approaches (Toyota et al. 2020). However, 3-dimensional (3D) morphometric studies that can analyze the shape of crustaceans are still lacking. Here, we constructed 3D images of the carapace of adult *E. isenbeckii* with an optical 3D scanner, then attempted to identify novel morphological indices to characterize and distinguish sexes. 3D-based approach will contribute the understanding the relation between genetic variation of population and morphological characteristics, and the developmental processes of sexual dimorphic traits in the horsehair crab.

Materials and Methods

Sample collection and imaging of carapaces

A total of 84 sexually-matured crabs consisting of 38 females and 46 males were collected in Funka Bay, Pacific Ocean, Southern Hokkaido, Japan in April 2019 (Toyota et al. 2020), and 32 females and 39 males with a complete carapace shape were used for this study. Fishing permission for horsehair crabs for this study was granted by the Hokkaido Governor. Prior to imaging, all carapaces were completely dried. 3D imaging of carapaces was conducted by non-contact method using light cutting method with a 3D scanner (VL-300, Keyence, Osaka, Japan) because no damages were induced during measurement. The time

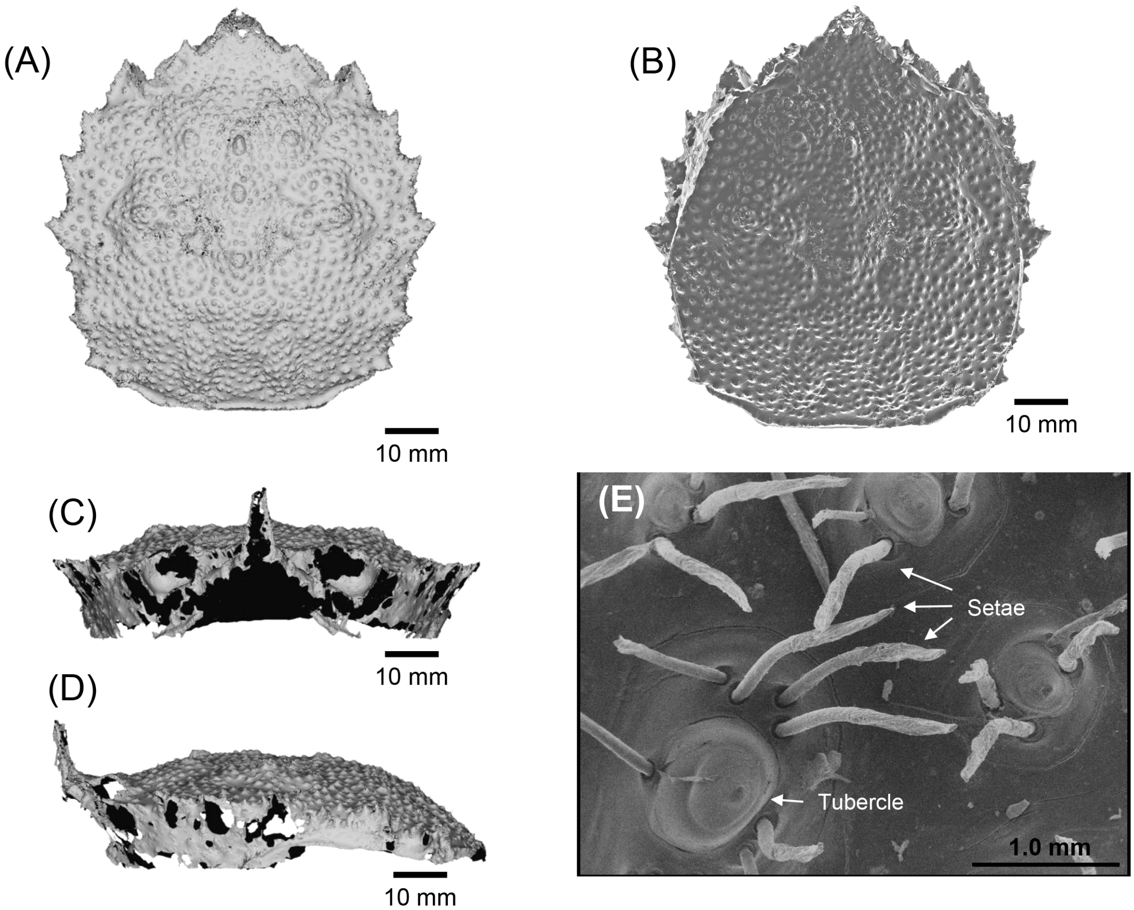

Fig. 1. 3D imaging of the carapace of the horsehair crab *E. insenbeckii*. Dorsal (A), ventral (B), frontal (C), and lateral views (D). SEM imaging of the setae around the tubercle on the carapace (E).

required for a scan is 2-3s. The resolution of the 3D scanner is ~0.1μm and it is enough to evaluate the structures of carapaces. Resultant structures of the carapace were reconstructed as standard triangulated language (STL) models, which is a 3D-computer aided design (CAD) model of the carapace. In terms of scanning electron microscope (SEM) imaging, carapace samples were dried up and cut into 1.0 cm square, and then scanned at 22-fold magnification by a JCM-5000 NeoScope (JEOL Ltd., Tokyo, Japan).

Counting tubercles on the carapace

The number of tubercles on the carapace (N_{bp}) was counted in female and male samples (Fig. 1E). First, STL files acquired in accordance with the method mentioned above was loaded with 3D-CAD software (Free CAD, the FreeCAD Team, https://www.freecadweb.org/). Second, the surface containing all tubercles seen from directly above was extracted as an image file (.tiff file). Finally, all tubercles at the image file were marked by using an image processing software (photoshop 2020, Adobe Inc., USA) and counted manually (see also Fig. 2B). For the area of the carapace (A_c), the image file acquired from STL file was loaded to an image processing software (ImageJ, National Institute of Health, USA) and A_c was defined as the area seen from directly above the carapace. To evaluate differences between sexes, the density of tubercles on the carapace (ρ) was defined using N_{bp} and the area

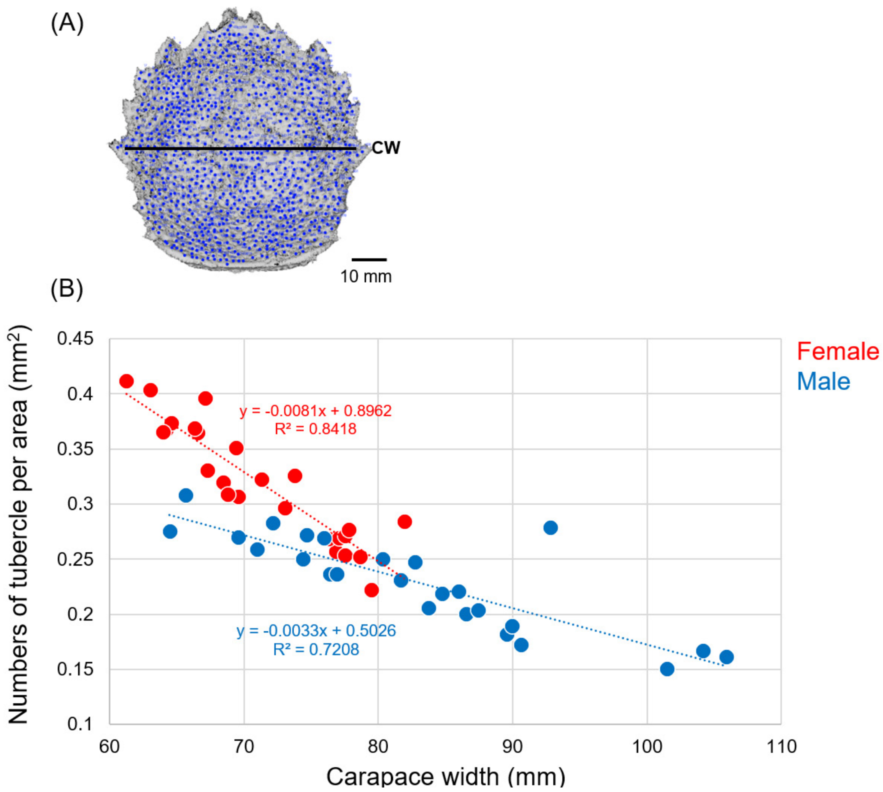

Fig. 2. Dorsal view of 3D scanned image of the carapace with marks of a tubercle (blue-dotted) (A). Black line indicates the carapace width (CW). Relationship between the number of tubercles per area on the carapace and carapace width between females and males (B). Red and blue dotted lines indicate the regression lines of females and males, respectively.

of the carapace (A_c), as follows:

$$\rho = \frac{N_{\mathrm{bp}}}{A_{\mathrm{c}}} \tag{1}$$

ρ is a parameter that is applied to characterize damage in ceramic material, and is well known as "crack density". By using this parameter, differences in N_{bp} can be quantitatively evaluated because it normalized by A_{c}. To estimate sexual differences in the densities of tubercles on the carapace, homogeneity of the slopes of the regression lines was tested by ANOVA using R software version 3.5.3 (R Core Team 2019).

Measurements of curvature radius of the carapace

As a 3D parameter to recognize sexual differences, the 3D CAD model of the carapace was virtually cut by a line corresponding to the width of CW. The curvature radius of the carapace was measured using the cut surface. The surface of the carapace is rough owing to the existence of tubercles. In this study, we focused on expressing the overall curvature without considering the roughness of the shell. Therefore, the radius of curvature was measured by approximating it as a part of a sphere, so the curvature radius (r_c) was expressed by the following equation:

$$r_c = \frac{0.5 \times (\mathrm{CW})^2 \times h}{2h} \tag{2}$$

h in Eq. 2 shows the distance between the chord of the carapace and the top of the carapace in a cut section (see also Fig. 3A). To estimate sexual differ-

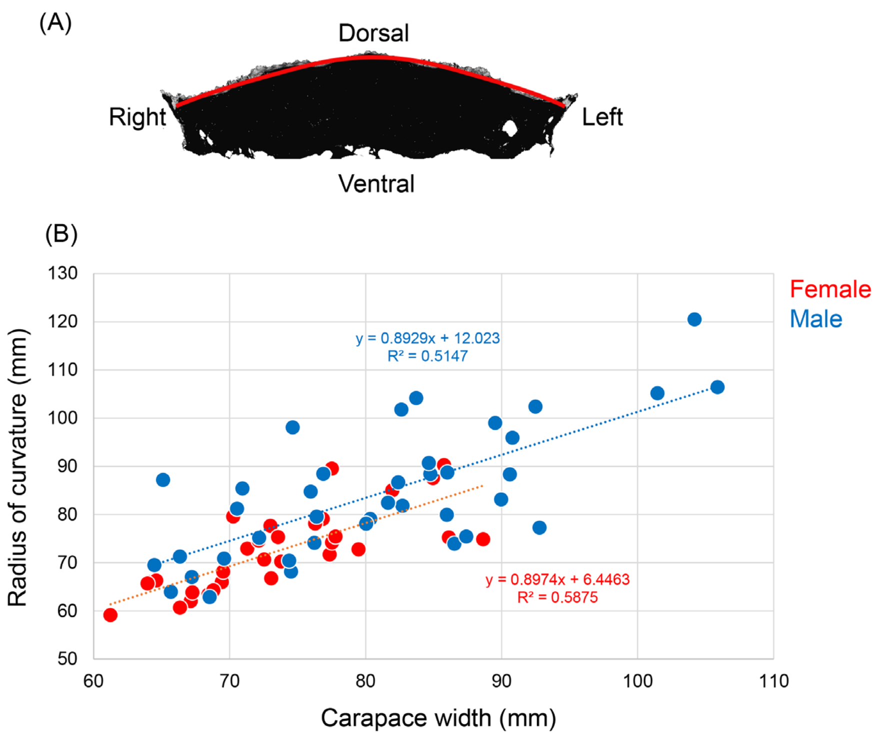

Fig. 3. Frontal view of 3D scanned images of the carapace (A). Relationship between the radius of curvatures of the carapace and carapace width between females and males (B). Red and blue dotted lines indicate the regression curves of females and males, respectively.

rences, analysis of covariance (ANCOVA) was performed with CW as a covariate using R software version 3.5.3 (R Core Team 2019).

Results and Discussion

Sexual differences in the density of tubercles on the dorsal surface of the carapace

Scanning and reconstruction of carapace 3D imaging were successfully conducted, enabling to get various parameters such as length, angle, number of specific structures, or something from every angles (Figs. 1A-D). The function of the tubercles with setae on the carapace is largely unknown, however similar structures are found in other cheiragonid brachyuran helmet crabs such as *Telmessus acutidens* and *Telmessus cheiragonus*. According to established setal classification, a seta can be defined as "an articulated cuticular extension of virtually any shape or size" (Watling 1989), and the setae on the *E. isenbeckii* carapace described here belong to the macrosetal system (several hundred micrometers, forming groups visible to the naked eye) (Jacques 1989). Unlike the British spider crabs, which have various setal types such as "hook-shaped" and "denticles (non-articulated cuticular outgrowth of the setal shaft)" on the carapace (Szebeni and Hartnoll 2005), the setae on the *E. isenbeckii* carapace are all similar (Fig. 1E). Moreover, three to ten setae formed a cluster around tubercle (Fig. 1E). To investigate the sexual differences in the density of tubercles (Figs. 2A and 2B), relationship between the density of tubercles and carapace width was investigated by comparing the slopes of the regression lines. No homogeneity between females and males was found ($F = 30.7$, $p < 0.01$), indicating that there is a clear difference between females and males in the densities of tubercles (Fig. 2C). In terms of the smallest carapace group (around 65 mm in carapace width), females had a higher density of tubercles than males. Although the fine details are in dispute, several biological functions of carapace setae and tubercles in *E. isenbeckii* have been considered, including acting as a physical sensor that detects changes in water flows or as a protecting cover camouflaged by mud and debris, and thus used as a reinforcement for the carapace. Although no substantial evidence of sexual differences in the setal system is available, our data indicates that smaller sexually-matured females have denser setal clusters on the dorsal surface of the carapace than males, leading speculation that smaller females might be more sensitive to environmental changes in their habitat.

Comparison of carapace curvature radius between females and males

It is accepted that the carapace of females is rounder than that of males in horsehair crabs (Hokkaido Research Organization). However, those morphological differences have not been quantitatively demonstrated, so we applied a 3D-based morphometrics approach in an attempt to clarify these differences. Although curvature radius is generally used to validate and re-define bone morphologies based on 3D models (Bachoura et al. 2013; Stagni et al. 2005), no applications have been reported for invertebrates. The radius of the curvature between females and males showed homogeneity in the slopes of the regression line against CW ($F = 0.204$, $p = 0.65$), so ANCOVA was performed with CW as a covariate. Consequently, a statistically significant difference between sexes was observed ($F = 3.22$, $p = 0.077$) (Fig. 3B). This data suggests that females have a rounder carapace than males, which may be the first evidence to quantitatively show sexual differences in the carapace based on 3D imaging.

Most brachyuran species display apparent morphological sexual dimorphism in the shapes of the chela, pleon, and/or carapace. To date, for quantitative validation of those traits, most studies have employed 2D-based geometric morphometric approaches using various crabs (Kalate et al. 2018; Grinang et al. 2019; Toyota et al. 2020). In the landmark-based method, all points (landmarks) are selected, and these are recognizable and homologous across all specimens, prior to the experiment (Bookstein 1997; Mitteroecker and Gunz 2009). Although such a landmark-based approach has been used successfully in many studies, it is often problematic, for instance, some landmarks lack fragile and/or complex objects such as spherical or rounded structures (e.g., carapace and claw). To resolve this technical issue, recent morphometric methods have enabled a more precise capture of curvatures and surfaces using a semi-automated landmarking procedure (Felice and Goswami 2017) and generalized Procrustes surface analysis (Pomidor et al. 2016). Moreover, the spherical harmonics-based morphometrics approach demonstrated that the shape of hermit crab claws can be evaluated precisely (Ege et al. 2020).

In conclusion, the current study provides new validating methods to distinguish female and male horsehair crabs using 3D-based morphometric approaches. Our methods may be applicable to the juvenile stage (< 60 mm CW), fossils, and stomach contents that female or male cannot be distinguished by abdominal morphology, geographic comparisons of carapace shape of horsehair crabs, and other brachyuran species. Indeed, in the fossil cheiragonid species, carapace is better preserved than the abdomen (Schweitzer and Salva 2000). Furthermore, recent technological advances in 3D-based approaches have made available both hardware (e.g., X-ray microcomputed tomography, and optical 3D scanner) and software that can potentially enhance the precision, objectivity, reproducibility, and applicability of morphometric studies. In the near future, those 3D-based technique will definitely be a new standard for morphometrics, even in crustacean research.

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