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Comparison of the cleaning methods for the preparation of brachyuran crabs for scanning electron microscopy

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ABSTRACT

The study presents a comparison of the techniques used by researchers for cleaning carapaces of brachyuran crabs for SEM preparation. The methods were applied on the heavily encrusted spider crabs inhabiting the Suez Canal; *Hyastenus hilgendorfi* and *Schizophrys dahlak*. The techniques were judged for their effectiveness in the elimination of the different fouling materials such as mucus, detritus and epibionts. In addition, a step for the removal of large epibionts was introduced to the cleaning protocol and tested for efficiency. Crabs' integuments were prepared according to the two methods and SEM micrographs were taken. The study ranked one method as more effective and showed that the suggested cleaning step was quite destructive to the setal types present on the crabs' integuments. Recommendations for the best cleaning steps for heavily fouled crabs are given.

INTRODUCTION

The major advantage of the scanning electron microscope (SEM) in biological studies is to investigate the surface detail and morphology of specimens at very high resolution **(Bozzola, 2014)**. These specimens could vary from whole organisms several centimeters in size to individual cells grown in culture. Due to the tremendous depth of field available to the operator, SEM allows an "in-depth" study of those specimens with great topography. SEM studies usually examine the external features of a specimen, whereas in transmission electron microscopy (TEM) studies, intracellular exploration is the main focus (**Osman *et al*., 2020**). Despite how good an instrument could be, the quality of the material photographed and the images obtained will be affected by how specimens are prepared for examination **(Felgenhaur, 1987)**.

There are standard steps necessary to prepare any tissue for SEM which include; fixation, dehydration, drying, mounting, and coating with palladium or gold **(Bomblies *et al.*, 2008)**. Samples for SEM imaging need to be dried as a final step in order not to

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disturb the vacuum in the microscope **(Pandithage, 2012)**. Drying also protects the samples from being collapsed or from any deformation in the structures under investigation as a result of water molecules. There are two standard methods for drying; air and critical point **(Bhattacharya *et al.*, 2020)** . In the air-drying method, nano and microstructures of the specimen can be affected by the tangential forces caused by the surface tension during crossing the interfaces from the liquid to the gaseous phase. On the other hand, in the critical point method, the physical characteristics of gas and liquid are not distinguishable which will preserve the sample morphology **(Pandithage, 2012)**.

The study of gross morphological detail in addition to fine structures of crustaceans have been considerably improved by the several recent technological advances in scanning electron microscopes (SEM). However, one of the main problems in preparing crustaceans, particularly brachyuran crabs, for the SEM is the removal of mucus as well as accumulated detritus. Another problem is the encrustation of their carapaces with several epibiotic organisms, ranging from symbiotic mites to bacteria and fungi. These organisms cause difficulties in the identification of the setal types and could obscure significant structures such as the terminal pores of chemoreceptors **(Bauer, 1975; Holmquist, 1985; Felgenhauer, 1987)**.

The integuments of the spider crabs inhabiting the Suez Canal are encrusted with excessive epibionts which make them remain totally inconspicuous in their habitat **(Sallam *et al.*, 2007; Madkour *et al.*, 2012)**. The encrusting cover has been reported to comprise of detrital material as well as two categories of epibiota: organisms that have been originally transplanted by the crab such as ascidians, sponges, and algae in addition to tube worms, barnacles, and bivalves that must have settled on the integument as larvae **(Sallam *et al.*, 2007)**. Together with detritus, micro-epibionts found on the integuments of both species are the major contributors to the mucus extracellular polysaccharide matrix covering the host's exoskeleton whose function is to facilitate settlement of macro-epibionts **(Madkour *et al.,* 2012; Osman *et al.,* 2015)**. The settlement of these macro-epibiota is enhanced by the thick and profuse layer of detritus that settle passively on the exoskeleton **(Sallam and Wicksten, 2011)**.

The most common method used for the removal of the mucus, bacteria and fungi as well as detrital material and other epibionts from the exoskeleton of the brachyuran crabs for their preparation for SEM examination is that by **Felgenhauer (1987)** in which chemical compounds were mainly used for the cleaning process. However, **Szebeni and Hartnoll (2005)** introduced a new method where specimens were cleaned by immersing crabs' carapaces in a detergent powder solution at a relatively high temperature. **Berke and Woodin (2009)** used another method for the removal of the decorating materials by means of forceps and brushes.

The present work has two objectives; first is to compare between the methods used for cleaning crabs' carapaces from fouling materials for SEM preparation. The second is to test the efficiency of a step in the cleaning protocol that could help with the

removal of the large epibionts covering the integuments. Methods will be applied on two species of spider crabs inhabiting the Suez Canal and judged for their effectiveness.

MATERIALS AND METHODS

Specimens of *H. hilgendorfi* and *S. dahlak* were collected from the Suez Canal at Timsah Lake. Since diving and underwater photography is illegal by law in the Suez Canal, crabs were obtained from fishermen using trammel nets with an approximate mesh size of 33.5 mm that are dropped at sunset and collected at dawn. Size of the crab is limited by the trap size; very small crabs were likely to escape from the mesh or not enter the trap at all. Crabs were examined for their molting stage, full intermolt adults ranging from 17.5-26.6 mm CW, 27.1-44.5 mm CW for *H. hilgendorfi* and *S. dahlak*, respectively were selected for examination.

Subsamples of 20 females and 20 males from both species were chosen in order to account for any variation in sex. Crabs were first fixed in 4F1G (Fixative, phosphate buffer solution) pH 7.4 at 4°C for 24 hours. We first tried the cleaning technique for the elimination of debris, epibionts and mucus by **Felgenhauer (1987)** but some carapaces were not entirely cleaned with visible amount of debris on them. The method by **Szebeni and Hartnoll (2005)** was subsequently applied, however, carapaces of female *H. hilgendorfi* were observed to be still fouled. The technique by **Berke and Woodin (2009)** for the removal of macro-epibionts by means of brushes and forceps was then applied. Yet, encrusting materials of our species strongly adher to the integuments and could not be easily dislodged. We therefore attempted to de-mask some of the females by separating the mask from the carapace. The mask was uplifted at the posterior border by means of a scalpel and removed carefully (Fig.1A, B). The exposed carapace was then treated as mentioned above.

We prepared the setae at least twice using both air and critical point drying method to avoid dehydration or shrinking of setal structures. Then carapaces were mounted using carbon paste on an aluminum stub and coated with gold up to a thickness of 400 A in a sputter – coating unit (JFC-1100 E). A JEOL JSM-5300 Scanning Electron Microscope (SEM) was used to image and characterize the surface morphology of coated exoskeleton samples.

RESULTS AND DISCUSSION

The method given by **Felgenhauer (1987)** was the one frequently used and most recommended by researches for preparing crustaceans for the SEM. In this method the author provided different procedures for the removal of debris and epibionts and mucus. However, when applied to our samples, the images obtained showed highly fouled structures (Fig. 2). The images showed the presence of a very thick layer of accumulated

detritus or debris (Fig. 2A, C, D, F, G, H& I), mucus (Fig. 2B) as well as epibiotic organisms (Fig. 2E) that caused difficulties in recognizing the different setal types. In contrast, this method gave successful results with the setae present on crustaceans' mouthparts **(Garm, 2005; Garm *et al.*, 2005)**, antennae **(Weisbaum and Lavalli, 2004)**, pereiopods **(Lavalli *et al.*, 2018)** and grooming appendages **(Wortham and LaVelle, 2016; Wortham and Pascual, 2019)**.

The method introduced by **Szebeni and Hartnoll (2005)** included a new cleaning technique that was applied on the carapaces of 7 species of decorating spider crabs. Although the authors showed that this technique was quite effective in removing the masking material of those species, **Salazar and Brooks (2012)** followed the method by **Felgenhauer (1987)** in the study of the setal morphology of the decorator spider crab *Microphrys bicornutus*. Their obtained images were clear enough for the setal types to be easily identified. Apparently, the last two studies are the closest to ours since they focused on the setal morphology of decorator crabs' carapaces. The species studied by **Salazar and Brooks (2012)** might have acquired a lower degree of fouling than ours which made the cleaning technique by **Felgenhauer (1987)** entirely effective in the elimination of debris and epibionts.

On that basis, we tried the cleaning technique by **Szebeni and Hartnoll (2005)**. The results this method displayed were fairly successful for our heavily fouled carapaces and allowed us to obtain good images (Fig. 3 A-D). The images showed cleaned carapace surfaces and distinguishable setal types. The various enzymes in the detergent powder together with the exposure to a relatively high temperature removed most of the encrusting materials without damaging the setae. However, not all of the carapaces were entirely cleaned, particularly those of females *H. hilgendorfi*. We therefore applied the cleaning technique by **Berke and Woodin (2009)** used for the decorator crab *Oregonia gracilis* but it was totally infeasible since the encrusting material were glued to the integuments of the crabs. Organisms such as barnacles, tube worms and ascidians overlap heavily on top of each other on the carapaces of our crabs in a way that renders them extremely difficult to separate with the use of forceps and brushes only. In addition, removal of these cemented materials requires some force which would definitely make this process very abrasive. This technique was appropriate in the case of *Oregonia gracilis* because most of the decorating materials used by this crab were organisms that could be detached easily, such as red and green algae, bryozoans and hydroids. Alternatively, and as an additional step before applying the method by **Szebeni and Hartnoll (2005)**, we de-masked the crabs that were not properly cleaned. De-masking was effective in removing all the large epibionts, however, SEM examination showed that this step was too destructive and that most of the setae were removed or damaged (Fig. 3 E&F).

Most of the authors who studied crustaceans' setal structures prepared their samples using the critical point drying method in order to avoid shrinking or dehydration.

Others such as **Wortham and LaVelle (2016)** and **Wortham and Pascual (2019)** used the air-drying method for all the body regions except for the gills which were critically-point dried. We therefore prepared the carapaces at least twice using the two drying methods. Results showed no distinguished differences in the setal morphology of our crabs by both methods as the different setal types kept their proper form and appeared without major artifacts (Fig. 3 G&H).

CONCLUSION

The preparation of fouled spider crabs' carapaces for SEM imaging requires specific cleaning in order to successfully remove the encrusting materials without damaging the setae. The results of this study showed that the cleaning method by **Szebeni and Hartnoll (2005)** was more effective in cleaning the heavily encrusted carapaces of spider crabs than that of **Felgenhauer (1987)** and **Berke and Wooden (2009)**. On the other hand, the step of de-masking proved to be rather destructive and totally ineffective in keeping the setae intact. The information reported herein could be useful to researchers who study spider crabs with similar carapace characteristics. Finally, and for best results, we recommend the use of fresh fully intermolt adults when available. They should be fixed immediately using the SEM basic preparation method then cleaned following the technique provided by **Szebeni and Hartnoll (2005)**.

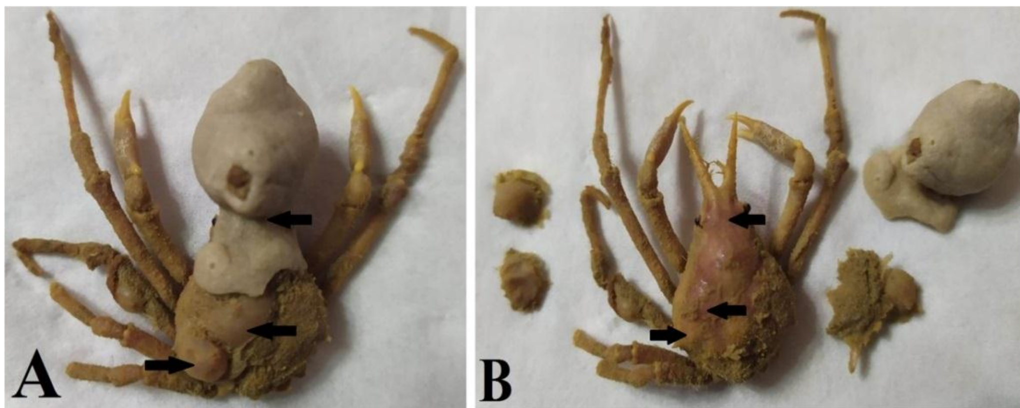

Fig. 1. (A); Carapace of *H. hilgendorfi* encrusted with ascidians indicated by arrows. (B); Same specimen after de-masking. Arrows point to cleaned regions of carapace.

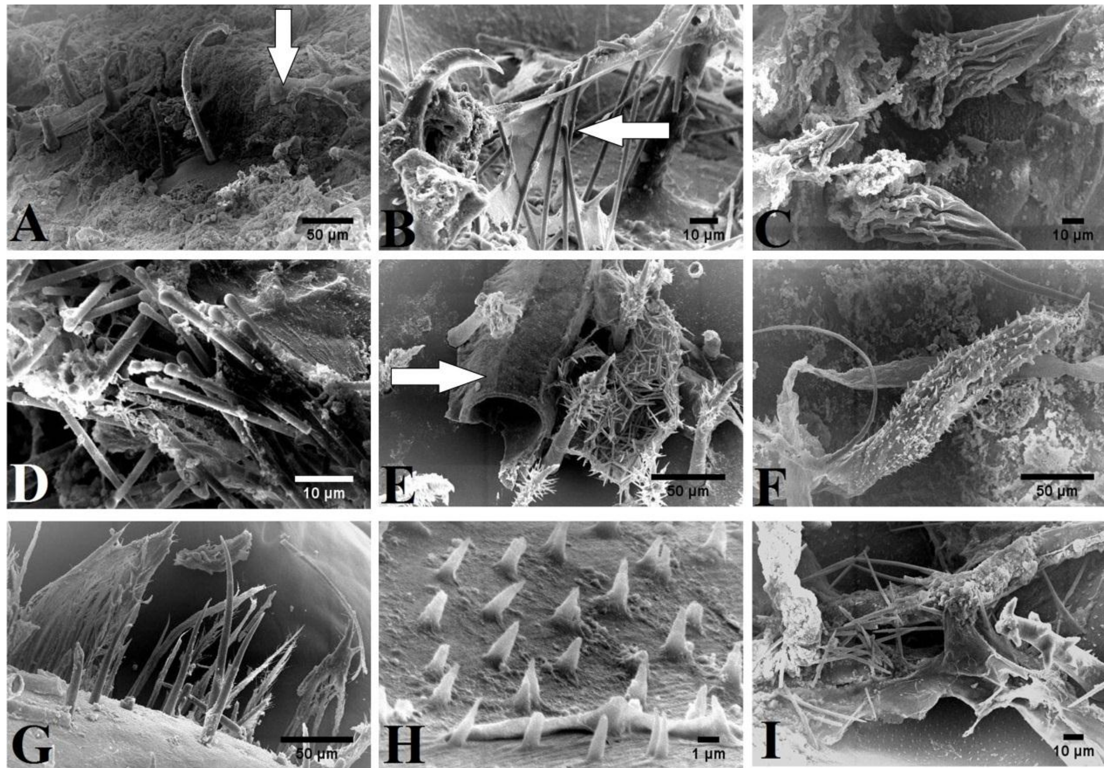

Fig. 2. *S. dahlak and H. hilgendorfi*: Heavily fouled areas on carapace. (A); Thick layer of debris indicated by arrow. (B); Arrow pointed to mucus. (E); Epibiotic organism indicated by arrow.

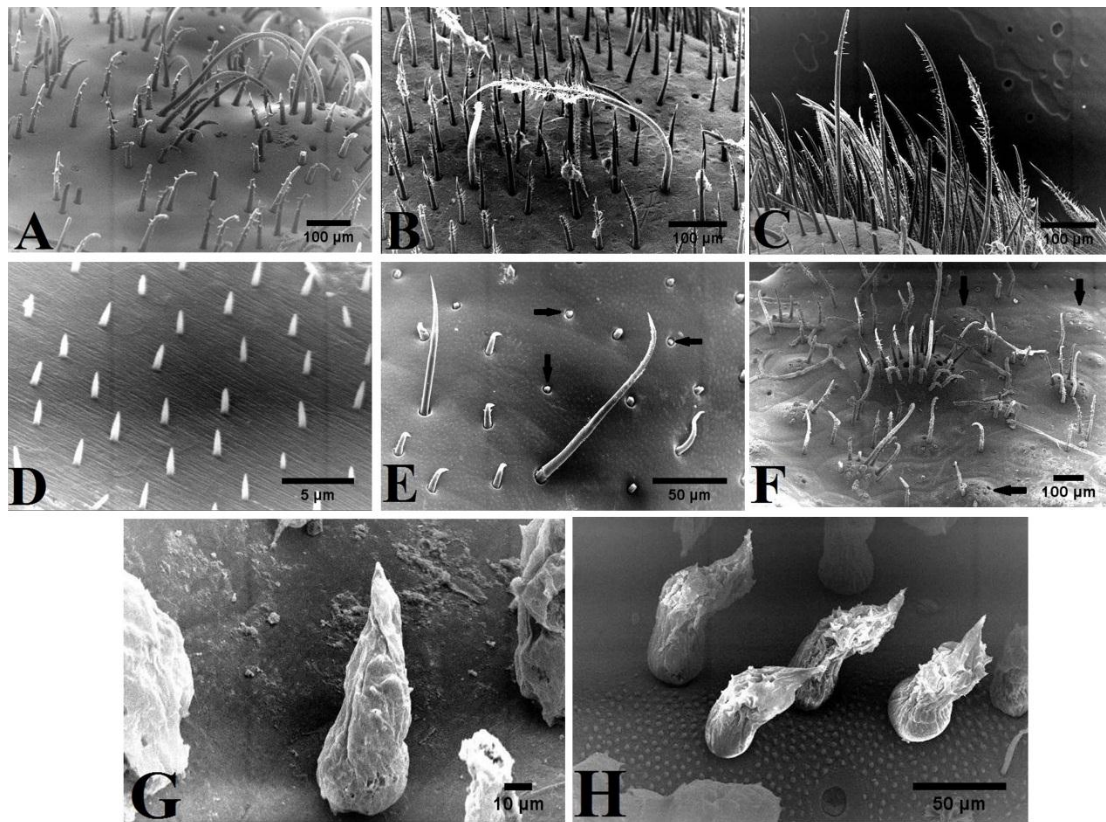

Fig. 3. (A-D); Different areas of cleaned carapaces showing various shapes of setae of *S. dahlak* and *H. hilgendorfi*. (E&F); Broken setae indicated by arrows on the carapace of *S. dahlak* carapace**.** (G&H); Same setal type observed in *H. hilgendorfi* prepared by air and critical point drying.

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