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Comparative ultrastructure study on the sperm morphology of two grapsid crabs

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ABSTRACT

The study envisaged the spermatozoon morphology of two grapsid crabs, *Grapsus albolineatus* and *G. tenuicrustatus* (Family: Grapsidea) via, electron microscope. The marked spermatozoa similarities between these two species appear to indicate a close phylogenetic proximity with the Grapsidae family. With few variations such as the absence of acrosome ray zone and thickened ring in the posterior acrosome, the spermatozoa of these two crabs display the features of thoracotreme synapomorphy. The distinct variations between these two species are the presence of onion ring in the peripheral outer acrosome layer of *G. tenuicrustatus*, and capsular flange in *G. albolineatus*. Apart from the other thoracotreme characters, the spermatozoa of these two crabs possess a protective outer sheath with striking differences.

Key words: *Spermatozoa, crustaceans, brachyurans, Grapsus albolineatus, Grapsus tenuicrustatus*

INTRODUCTION

Spermatozoon morphology has been used in recent decades as a phylogenetic criterion in crustaceans (Hinsch, 1973; Jamieson, 1989 a,b 1990; Jamieson and Tudge, 1990; Klaus *et al.* 2009; Ganapiriya, 2012; Sakunthala, 2014). Structurally the spermatozoa of many decapods such as, *Eriocheir japonicus* (Yasuzumi, 1960), *Callinectes sapidus* (Brown, 1966), *Petalomera lateralis* (Jamieson, 1990), *Portunus pelagicus* (Jamieson and Tudge, 1990), *Sesarma haematocheir* (Honma *et al.*, 1992), *Metopograpsus messor* (Anilkumar *et al.*, 1999), *Parasesarma plicatum* (Ganapiriya, 2012), *Sarmatium punctatum* (Sakunthala, 2014) are comprised of spherical acrosome fitted into a cup-shaped nucleus and a thin cytoplasmic layer between the nucleus and the acrosome and radial arms. Jamieson (1994) analyzed the phylogeny of brachyuran crabs using 34 ultrastructural characters; 27 spermatozoal and 7 non-spermatozoal suggesting brachyurans as a monophyletic taxon, also been supported at molecular

Ultrastructural studies on decapod spermatozoa suggested species-specific variation evidencing its importance in the taxonomic and phylogenetic studies (Tudge, 1997). Considering the usefulness of the spermatozoal studies in animal taxonomy (Jamieson, 1991a; Medina, 1995; Jamieson *et al*. 1995; Jamieson and Tudge, 2000), the present work analyses and describe the characteristics and differences, if any, in the spermatozoa of *G. albolineatus* and *G. tenuicrustatus*.

MATERIALS AND METHODS

The grapsid crabs of *G. albolineatus* (Fig. 1) and *G. tenuicrustatus* (Fig. 2) of carapace width 5-8 cm and 5-9 cm respectively were collected from the rocky substratum of the intertidal regions of Kadiapattanam ($10^0$58'N; $78^0$31'E), and Muttom ($9^0$83'N; $76^0$71'E), Kanyakumari District, Tamil Nadu, India. The animals were captured using fish flesh as bait and were reared in the laboratory in plastic containers in near natural condition and fed with clam meat and egg white.

Transmission electron microscope (Walker, 1975)
The dorsal side of the carapace was cut opened and the vas deferens was dissected out carefully under a dissection microscope (COSLAB VS – 5) and the tissue (2×2 mm pieces) was fixed in Karnovsky's fluid (4% paraformaldehyde and 3% glutaraldehyde in phosphate buffer, pH 7.2). After 24 hours of fixation, the tissues were washed twice in phosphate buffer (pH 7.2-7.4) and in 1% osmium tetroxide for one hour at 4°C. The same was washed with phosphate buffer (pH 7.2 – 7.4) and transferred to stain (2% Uranyl acetate in 95% ethyl alcohol) followed by dehydration with absolute ethanol. Clearing of the tissue was done in propylene oxide and subsequently embedded in araldite and propylene oxide in the ratio 1:1. The tissues were infiltered with epoxy resin, and the blocks were prepared. Semi-thin sections of 1µm thickness were stained with Toluidine blue (1%) and mounted in DPX. Ultra-thin sections of the desired area (500A°) were stained with saturated aqueous uranyl acetate and lead citrate and examined and photographed under the Transmission Electron Microscope (EMI K5302 Emu.6).

Phylogenetic programs
The ultrastructural structure of spermatozoal matrix includes 14 binary and 13 multi-state characters. The spermatozoon multiple character coding data matrix ranges between 0 and 5 (Jamieson, 1994). Phylogenetic analysis of the spermatozoa characters of the candidate species is compared with other brachyuran crabs (10 in number) of different families was performed under the PAUP (Phylogenetic Analysis Using Parsimony) version 4.0 (Swofford, 1998) program. Maximum Parsimony (MP) (Durbin *et al*. 1998; Hall, 2004) uses a character based the algorithm and possible tree structures and assigning a cost to each tree. The exhaustive-search or the branch and bound method (Hendy and Penny, 1982) is used to study large data and evaluates all possible trees, but cutting off paths of the search tree that cannot lead to optimal trees. Bootstrapping aims to assign confidence limits to nodes on a tree or it randomly resampled characters to form data sets having the same number of characters as the original, then searches these sets for the most parsimonious trees and counts the number of times in which nodes occurring on the original tree also appear in the replicates. Bootstrap analysis 100 replications were made.

RESULTS and DISCUSSION

The brachyuran spermatozoa displayed diversity in shape and size even among the closely related species. Sperms of *G. albolineatus* (Fig. 3) and *G. tenuicrustatus* (Fig. 4) renowned all the diagnostic features of thoracotreme sperm such as *Uca dussumieri, Macrophthalmus crassipes, Mictyris longicarpa, P. plicatum* (Jamieson *et al*. 1995; Ganapiriya, 2012). The presence or absence of 27 spermatozoal characters and the character coding of candidate species was represented in Table 1 and 2 respectively. Among the twenty-seven characters, 23rd (concentric lamellae) and 26th (capsular flange) characters were incongruent in *G. albolineatus* and *G. tenuicrustatus*.

The acrosome is subspheroidal and trilayered (Fig. 3A and 4A) in both the candidate species akin to other brachyuran crabs such as *M. messor* (Anilkumar *et al*. 1999), *Maja brachydactyla* (Simeo *et al*. 2010) while bilayered in *Cardisoma carnifex* (Jamieson *et al*. 1996), *Neodorippe astuta* and *Portunus pelagicus* (Jamieson and Tudge, 1990), and *Odiomaris pilosus* (de Forges *et al*. 1997). Length and width ratio of the acrosome of *G. albolineatus* and *G. tenuicrustatus* was 0.8:1.1 and 1.9:1.2 respectively. Earlier reports revealed that the shape and L:W ratio of the acrosome is of phylogenetic significance. The L:W ratio would be in the range of 0.8-1.2 in heterotreme and thoracotreme brachyuran crabs.

Table 1. Comparison of the spermatozoa of *G . albolineatus* and *G. tenuicrustatus* (after Jamieson 1994).

S.No	Characters of spermatozoa	G. *albolineatus*	*G. tenuicrustatus*
1	Acrosome length/width	3.5μm	3.3μm
2	Zonation of the acrosome	Concentric	Concentric
3	Perforation of operculum	Perforate with apical button	Perforate with apical button
4	Opercular projection	Absent	Absent
5	Continuity of Operculum	Discontinuous	Discontinuous
6	Thickness of operculum	Moderate	Moderate
7	Operculum width	Not extremely wide	Not extremely wide
8	Periopercular rim	Less prominent	Less prominent
9	Accessory opercular ring	Absent	Absent
10	Subopercular protuberance	Absent	Absent
11	True acrosome ray zone	Absent	Absent
12	Outer acrosome zone	Not ragged	Not ragged
13	Antero lateral pale zone	Absent	Absent
14	Flange like lower zone	Absent	Absent
15	Xanthid ring	Absent	Absent
16	Subacrosomal chamber	Pre- equatorial	Pre- equatorial
17	Head of perforatorium	Round	Round
18	Lateral arms	Several	Several
19	Composition of lateral arms	Nuclear only	Nuclear only
20	Centrioles	Absent	Absent
21	Posterior median process of nucleus	Absent	Absent
22	Thickened ring	Absent	Absent
23	Concentric lamellae	Absent	Present
24	Capsular chamber	Absent	Absent
25	Capsular projection	Absent	Absent
26	Capsular flange	Present	Absent
27	Corrugation of perforatorial chamber	Absent	Absent

Table 2. Spermatozoal character coding matrix

Taxon	**1 1 1 1 1 1 1 1 1 1 2 2 2 2 2 2 2** **1 2 3 4 5 6 7 8 9 0 1 2 3 4 5 6 7 8 9 0 1 2 3 4 5 6 7**
Grapsus albolineatus	3 1 1 0 0 0 0 1 0 0 0 0 0 0 0 1 0 4 2 0 0 0 0 0 0 1 0
Grapsus tenuicrustatus	3 1 1 0 0 0 0 1 0 0 0 0 0 0 0 1 0 4 2 0 0 1 1 0 0 0 0

Fig. 1 Dorsal view of *Grapsus albolineatus*

Fig. 2: Dorsal view of *Grapsus tenuicrustatus*

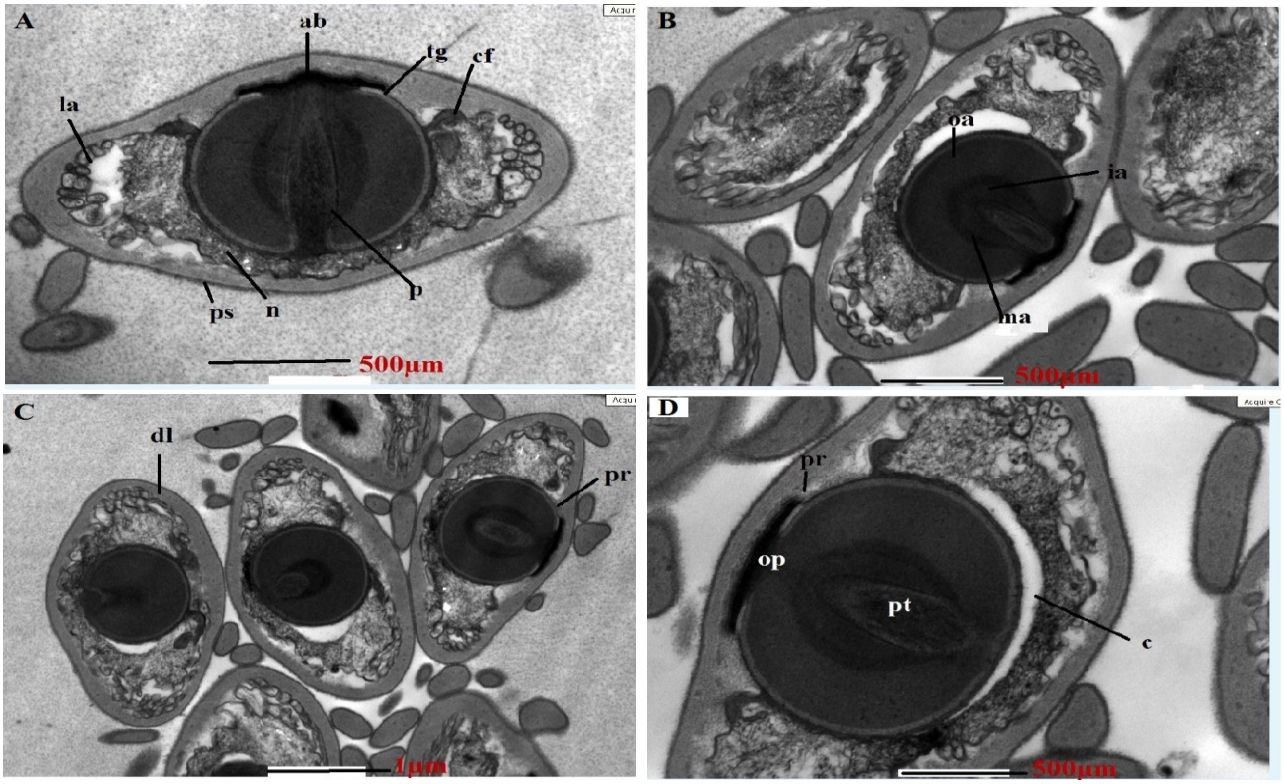


Fig. 3: Electron micrograph of the spermatozoa of *G. albolineatus.*

oa – outer acrosome, **ia** – inner acrosome, **ma** – middle acrosome, **tg** – tongue and grove connection, **p** – perforatorium, **pt.** perforatorial tubules, **n** – nucleus**, c** – cytoplasm, **m** – mitochondria, **pr** – periopercular rim, **ab** – apical button, **cf** – capsular flange, **dl** – double layer, **ps-** protective sheath, **op** – operculum, **la-** lateral arms.

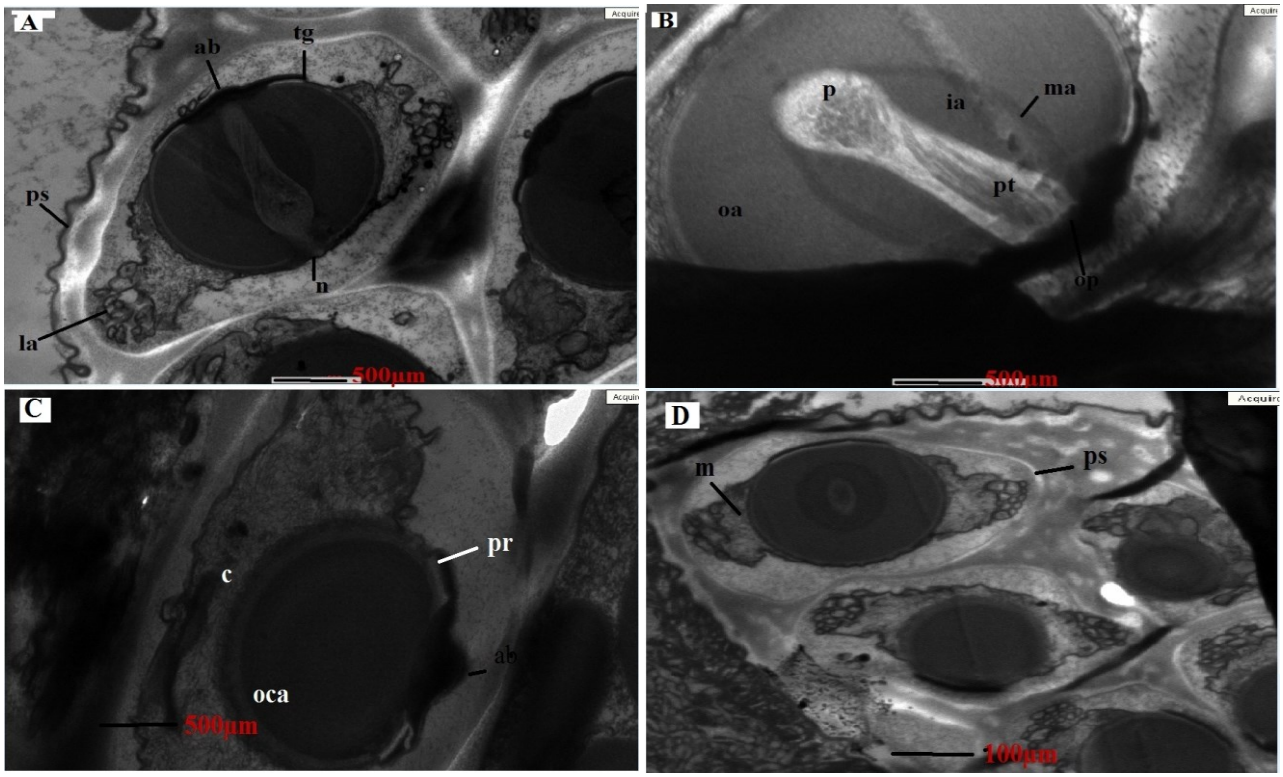
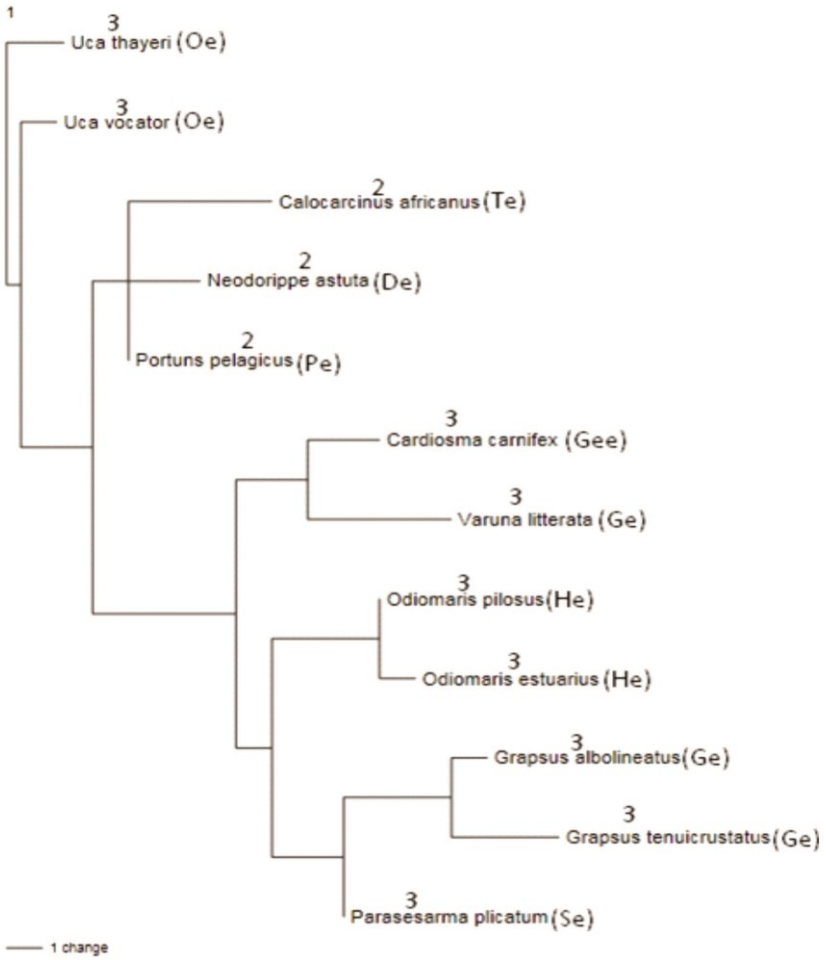


Fig. 4: Electron micrograph of the spermatozoa of *Grapsus tenuicrustatus*.

oa – outer acrosome, **ma** – middle acrosome, **ia** – inner acrosome, **oca** – outer concentric acrosome, **tg** – tongue and grove connection, **ps-** protective sheath, **n** – nucleus**, c** – cytoplasm, **p** – perforatorium, **pt.** perforatorial tubules, **m** – mitochondria, **pr** – periopercular rim, **op** – operculum, , **ab** – apical button



Oe – Ocypodiae; Te – Trapeziidae; De – Dorippidae; Pe – Portunidae; Gee – Gecarcinidae, He – Hymenosomatidae; Ge – Grapsidae; Se – Sesarmidae; 2 – Heterotremata; 3 – Throacotremata

Fig. 5 Maximum parsimony analysis

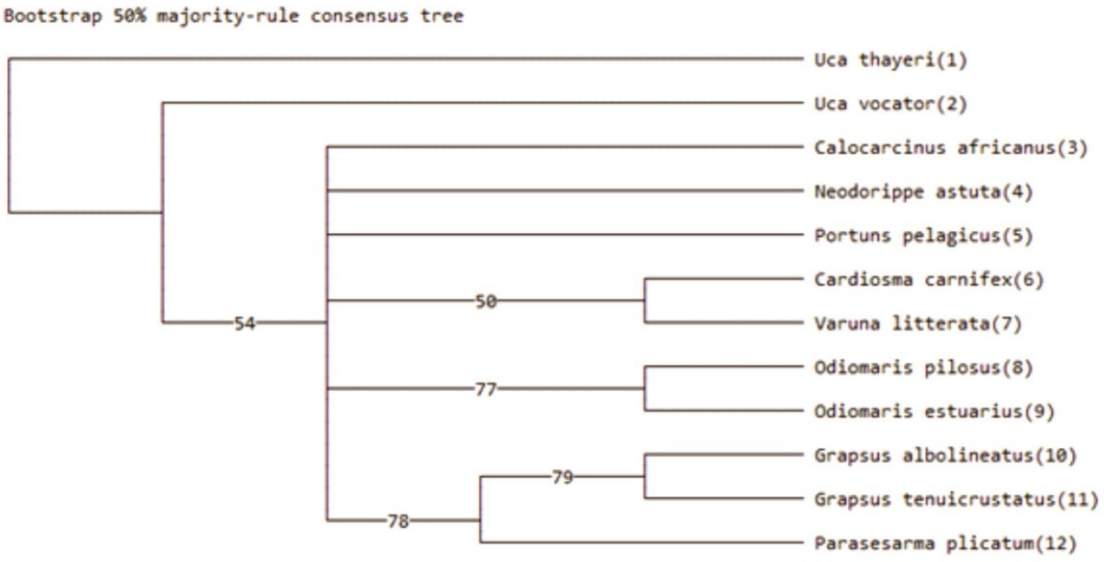


Fig. 6 Bootstrap analysis

The perforatorium (Fig. 3A and 4B) of the two candidate species was drawn-out from the posterior pole of the acrosome to the base of the operculum anteriorly, as the features pronounced for the thoracotreme (Jamieson *et al*. 1996; Cuartas and Sousa, 2007). In *G*. *albolineatus* and *G. tenuicrustatus*, the operculum is perforated and capped with an apical button that characterizes basal thoracotreme character (Fig. 3D and Fig. 4C). The apical button appears ambiguous to owe to its alternative absence or loss in thoracotremes such as *M. crassipes* (Jamieson, 1991a), *Varuna literata* (Jamieson *et al*. 1995) and *U. thayeri* (Benetti *et al*. 2008). Presence of oblique and horizontal accessory opercular ring is a typical thoracotreme character (Jamieson *et al*. 1996; Anilkumar *et al*. 1999; Ganapiriya, 2012) was absent in the two crabs under study. The occurrence of peri-opercular rim (Fig. 3C and Fig. 4C) in few Thoracotremata appears to be a feature that separates these species from the rest of the grapsid (Jamieson *et al*. 1996).

In the present study, the cytoplasm was reduced into a thin layer ensheathing the three by fourth of the outer surface of the acrosome vesicle (excepting the opercular region) and basally connected with the contents of the perforatorium chamber (Fig. 3D and Fig. 4C). The cytoplasm contains mitochondria and few membranous structures as reported in other brachyuran crabs (Tudge *et al*. 1998; 2001; Ganapiriya, 2012). In *G*. *albolineatus*, the nucleus was cup-shaped to surround all the lateral, anterolateral and posterior surfaces of the acrosome vesicles (Fig. 3A). Alternatively, in *G. tenuicrustatus*, the nucleus appeared wing-like and much reduced on the posterior side (Fig. 4A). The nuclear material in both the species is diffuse, and the chromatin assumes a filamentous form as described in few other grapsid crabs (Anilkumar *et al*. 1999; Ganapiriya, 2012; Shylasuganthi. 2015) and *Geryon* species (Hinsch, 1988). In the current study, several nuclear arms or spikes seems to be originated from the nucleus containing chromatin microtubules and arranged over the equatorial plane of the acrosome vesicle. The number of the arms is variable and characteristic for each species which either contains microtubules or mitochondria (Fig. 3B and 4D) (Medina and Rodriquez, 1992a; Cuartas and Sousa, 2007; Ganapiriya, 2012).

The protective sheath (envelope) encompassing individual spermatozoa within the spermatophores of *G. albolineatus* (Fig. 3A) and *G. tenuicrustatus* (Fig. 4D) invites considerable interest. The envelope exhibits striking difference in such a way that the sheath of *G. tenuicrustatus* was thin enclosing a homogeneous granular matrix, where the spermatozoon was embedded. Nevertheless in *G*. *albolineatus*, the medium electron dense sheath was double layered with concentric lamellations (Fig. 3C) encompassing the spermatozoa within flimsy granular matrix. Previously it was stated in two other freshwater brachyuran *Parasesarma* species (Ganapiriya, 2012; Shylasuganthi, 2015). The sheath probably protects and nourishes the spermatozoa while it is stowed within the reproductive tract for long time. The exact significance of possessing such sheath was uncertain and yet to be analyzed.

The topology of the spermatozoan character analysis was based on a data set comprising 12 taxa from different families. 100 bootstrap replicates support branch differentiation from other taxa on the strict consensus tree (Fig: 5). Five internal nodes were also supported by strong bootstrap values in MP analysis. Phylogram inferred from spermatozoa morphology revealed five clades with tree length 38 and Consistency Index (CI) 0.6316. All nodes within this clade have a homoplasy index (HI) of 0.3684. Heuristic search settings shown the characteristics summary of 27 spermatozoa charters hold equal weight and 'unord type' with four parsimony-uninformative characters and 14 parsimony informative characters. For the reduced set of taxa, the bootstrap method with heuristic search for 100 replications contributed 50% majority rule consensus tree of length 40; (CI) - 0.632; Homoplasy Index (HI) - 0.368; Retention Index (RI) - 0.667; Rescaled Consistency Index (RC) - 0.421; f value - 54; f ratio - 0.1843. 27 characters are resampled in each replicate (Fig. 6). All characters are the 'unord' type and it has equal weight and bootstrap values are conservative estimates of significance. Thus the two grapsids *G. albolineatus* and *G. tenuicrustatus* associate in 79% of trees. Parsimony analysis of the spermatozoal character of our candidate species was examined with 10 other brachyuran crabs on different families. Of these Gecarcinidae, Hymenosomatidae, Grapsidae belongs to Thoracotremata, which forms a separate clade. The heuristic (the branch and bound and bootstrap) search revealed that the Grapsidae associates 78%, while Hymenoptera associates 79% and Gecarcinidae 50% (Fig. 6).

To conclude, spermatological characters has been used by several authors to investigate the taxonomic and phylogenetic status of a number of brachyuran crabs to

trace the existence of the species in question in the three higher groups of brachyurans (Jameison, 1991; 1994; Anilkumar *et al*. 1999; Ganapiriya, 2012). Our studies revealed that the two crabs belong to thoracotreme (based on acrosome zonation, operculum, peri-opercular rim, the absence of acrosome ray zone) though they share some of the heterotreme characters. At this juncture, it worth to recall the reports of Jamieson (1994) and Guinot *et al*. (1994), who stated Podotremata as a monophyletic taxon and Heterotreme and Thoracotreme as sister groups.

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