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Original Article

Morphological investigation and analysis of ribosomal DNA phylogeny of two scale-worms (Polychaeta, Polynoidae) from the Gulf of Thailand

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Abstract

Scale-worms are polychaetes of the family Polynoidae that are commonly distribute in marine environments. This study aims identify and introduce two scale-worms as *Capitulatinoe* cf. *cupisetis* and *Eunoe* cf. *oerstedi* from the western coast of the Gulf of Thailand. Using scanning electron microscopy of adult worms, the antennae, palps, prostomium, cirri, setigers, parapodia, saetae and elytra are described. In addition, the phylogenetic relationships of our specimens with other polychaete species were analyzed based on partial sequences of 28S, 18S and 16S ribosomal DNA (rDNA) genes. The rDNA sequences identified *C.* cf. *cupisetis* and *E.* cf. *oerstedi* were respectively recovered within Arctonoinae and Polynoinae in a monophyletic Polynoidae. The congruence or incongruence of the morphological and molecular data is discussed in the text. These findings increase the knowledge of polynoid polychaete worms in Thailand, although two scale-worms remain to be identified of the precise species.

Keywords: *Capitulatinoe* cf. *cupisetis*, *Eunoe* cf. *oerstedi*, the western coast

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1. Introduction

Scale worms are free-living, carnivorous polychaetes belonging to the family Polynoidae (suborder Phyllodocida) and were originally described by Kinberg (1856). They are widespread, occurring primarily in marine regions in tropical and subtropical environments, from shallow to deep seas (Fauchald, 1977). The family Polynoidae consists of about fifteen subfamilies, with more than two hundred genera recognised worldwide (Barnich *et al.*, 2012; Fauchald, 1977; Malmgren, 1867; Pettibone, 1969). Recently, studies of the family include Salazar-Vallejo *et al.* (2015) who reported the polynoid *Lepidasthenia loboi* as a new species from Puerto Madryn, Argentina and De Assis *et al.* (2015) who created the catalogue of the eighteen scale worms in genus *Lepidonotus* from South America. The genus *Capitulatinoe* Hanley and Burke 1989 belongs to the subfamily Arctonoinae and contains only one member, *C. cupisetis*. The *C. cupisetis* is a commensal organism inhabiting the ambulacral grooves of asteroids in Broome, Western Australia (Hanley & Burke, 1989). The genus *Eunoe* Malmgren, 1866 is a large genus belonging to the subfamily Polynoinae. It is distributed in a wide range of marine environments and contains more than fifty recognised species (Bellan, 2001); for example, *E. assimilis* from South Africa (McIntosh, 1924); *E. spinosa* from Sagami Bay and Sagami Sea, Japan (Imajima, 1997); *E. hydroidopapillata* from Kamchatka, Russia (Rzhavsky & Shabad, 1999); *E. tuerkayi* from the Adriatic Sea (Barnich & Fiege, 2003); *E. yedoensis* from the Arabian Peninsula (Wehe, 2006) and *E. nodosa* from the North Atlantic (Barnich & Fiege, 2010). *E. oerstedi* is one of the members of the genus originally recorded by Malmgren (1866) and inhabits the Western North Atlantic Ocean (Barnich & Fiege, 2010).

Ribosomal DNA (rDNA) sequences of the nucleus and mitochondria are commonly used molecular tools in taxonomy for identifying and defining large metazoan taxa (Canales-Aguirre *et al*., 2011; Machida & Knowlton, 2012). Moreover, Norlinder *et al*. (2012) evaluated the phylogenetic relationships within the scale-worms of the Aphroditiformia based on partial nucleotide sequences of nuclear genes and mitochondrial genes. Carr *et al*. (2011) provided an important phylogeny of polychaetes including *E. oerstedi* by using mitochondrial cytochrome c oxidase I (COI) from Canadian oceans, and 28S rDNA sequences of this species that were already deposited into GenBank (http://www.ncbi.nlm.nih.gov). Recently, Serpett *et al*. (2016) reported the excellent phylogenetics of *Eunoe* spp. and other polynoid species based on 18S, 28S, 16S rDNA and COI from the Southwest Indian Ocean Ridge. The aim of the present study was to report *C.* cf. *cupisetis* and *E.* cf. *oerstedi* from the western coast of the Gulf of Thailand and to describe the surface morphology of both species using scanning electron microscopy (SEM). In addition, the phylogenetic relationships of *C.* cf. *cupisetis* and *E.* cf. *oerstedi* within the polynoid families were assessed using partial DNA sequences of 28S, 18S and 16S rDNA.

2. Materials and Methods

2.1 Polynoid collections

Capitulatinoe scale-worms were found at two sites and *E.* cf. *oerstedi* at one site during a survey of intertidal polychaete worms over six sampling sites in the Gulf of Thailand, Prachuap Khiri Khan Province (Figure 1A). In the present study, polychaetes were surveyed from July to December 2013. *Capitulatinoe* cf. *cupisetis* were collected from the ambulacral grooves of *Astropecten indica* in areas of fine sand at 1-4 m depth, and *E.* cf. *oerstedi* were sampled from marine mud in the shells of dead *Pinna* sp. bivalves collected at 8–12 m depth using an Agassiz trawl (Figures 1B, and C). Sampled worms were placed in small aquaria con-taining natural seawater that were equipped with air pumps, and the aquaria were then transported to the laboratory. For subsequent specimen descriptions, the living worms were anaesthetised with 7% $MgCl_2$ in cool seawater and their general morphologies observed for species identification under a stereomicroscope. The specimens were then fixed in 4% formaldehyde for overnight and transferred into 70% ethanol for storage. The scale-worms were identified ac-cording to a key by Hanley and Burke (1989) for *C. cupisetis* and keys by Banse and Hobson (1974), Pollock (1998) and Barnich and Fiege (2010) for *E. oerstedi*. The nomenclature used to describe the morphology of polychaetes followed Rouse and Pleijel (2001).

2.2 SEM investigation

Adult individuals each of *C.* cf. *cupisetis* and *E.* cf. *oerstedi* were fixed overnight in 2.5% glutaraldehyde fixative in 0.1 sodium cacodylate buffer, pH 7.4, at 4 °C. The worms were then washed three times with 0.05 sodium cacodylate buffer and then post-fixed in 1% osmium tetroxide in 0.1 sodium cacodylate buffer, pH 7.4, at 4 °C for 1 h. They were then washed three times with distilled water and dehydrated through a graded ethanol series. After dehydration, the specimens were dried in a critical point drying machine (Hitachi HCP-2) using liquid carbon dioxide as a transitional medium. The specimens were mounted on aluminium stubs, coated with gold in an ion-sputtering apparatus (SPI-Model sputter coater) for 4 min and examined using a JEOL JSM-5400 scanning electron microscope operating at 15 kV.

2.3 Molecular phylogenetic analysis

Polynoid genomic DNA from *C.* cf. *cupisetis* or *E.* cf. *oerstedi* was extracted from each of fresh samples using a DNeasy Tissue kit (Qiagen) according to the manufacturer's protocol. The nuclear and mitochondrial genes were amplified using *Taq* DNA polymerase (Takara, Tokyo, Japan). The primer pairs for 28S rDNA amplification were 5' ACCC GCT GAATTTAAGCAT-3' and 5'-TCCGTGTTTCAAGACGG-3' (~800 bp) (Lê *et al*., 1993), for 18S rDNA they were 5'-TACCTGGTTGAT CCTGCCAGTAG-3' and 5'-GATCCTT CCGCAGGTTCACCTAC-3' (~1,750 bp) (Giribet *et al*., 1996) and for 16S rDNA they were 5'-CGCCTGTTTATCA AAAACAT-3' and 5'-CCGGTCTGAACTCAGATCACGT-3' (~450 bp) (Ruta *et al*., 2007). Polymerase chain reactions (PCR) were conducted at thermal cycling conditions involving an initial denaturation step at 95 ºC for 3 min, followed by 30 cycles of denaturation at 94 ºC for 30 s, annealing at 52 ºC for 30 s and extension at 72 ºC for 1 min. A final extension was performed at 72 ºC for 10 min. The PCR products were electrophoresed on a 1% agarose gel, stained with ethidium bromide, and viewed on a UV transilluminator. DNA

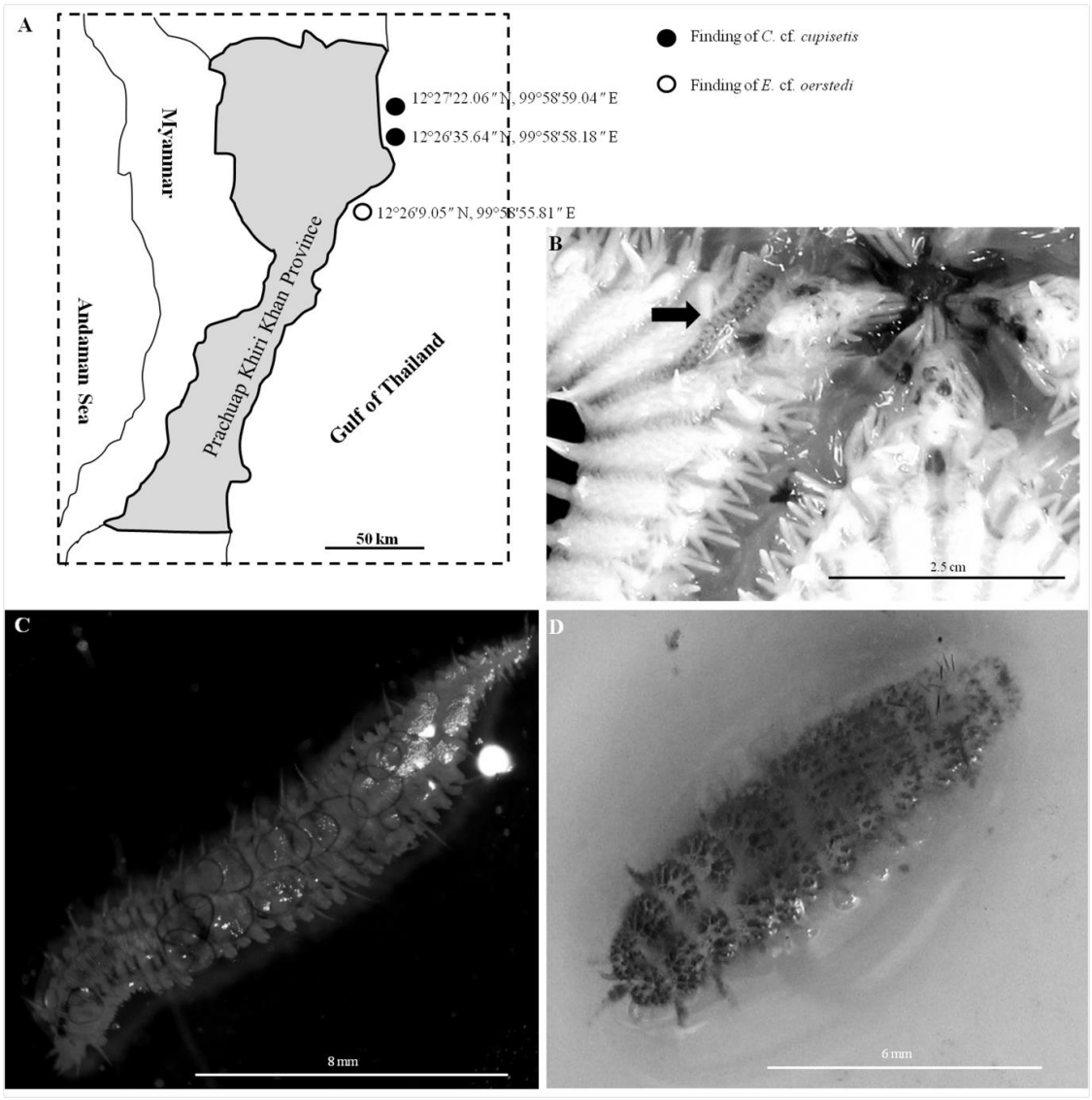

Figure 1. Study area including the sites of polychaete sampling from the western coast of the Gulf of Thailand (A). Circles indicate the sample localities where *C.* cf. *cupisetis* (B) and *E.* cf. *oerstedi* (C) were found.

fragments from the PCR products were purified using the QIA-quick Gel Extraction Kit (Qiagen) with spin columns and stored at 4 ºC. DNA sequencing was performed by Macrogen DNA Sequencing Service, Korea. The partial sequences of the *C.* cf. *cupisetis* and *E.* cf. *oerstedi* genes were deposited into GenBank (http://www.ncbi.nlm.nih.gov) under the accession numbers in Table 1. The DNA sequencing results were analysed for regions of local similarity using the online program Basic Local Alignment Search Tool (BLAST) (http://blast. ncbi.nlm.nih.gov/Blast.cgi). Multiple sequence alignments were performed with the Clustal Omega multiple sequence alignment program (McWilliam *et al*., 2013). For the phylogenetic examination of polychaetes, the bootstrap method with neighbor joining was conducted using the software package Molecular Evolutionary Genetics Analysis (MEGA) version 5.10 (Tamura *et al*., 2011) for Windows software.

3. Results

3.1 General polynoid morphology

In *C.* cf. *cupisetis*, the body of specimens prolongs subcylindrically, with numerous setigers covered by numerous pairs of elytra. The average body size is 16 mm long and 2 mm wide (Figure 1B). Prostomium is hexagonal oval, undivided into two lobes, two pairs of eyes, lacking cephalic peaks, three antennae, paired palps and paired tentacular cirri. Two lateral antennae are smaller and shorter than the median antenna. Tentacular cirri are long and present posterolateral to

Table 1. Lists of polynoids, other polychaetes and outgroup species with accession numbers of 28S, 18S and16S rDNA sequences.

Species	rDNA	Accession number	Reference
Ingroup			
Amphiduros fuscescens	28S	DQ442598	Ruta *et al*., 2007
	18S	DQ442584	Ruta *et al*., 2007
	16S	DQ442569	Ruta *et al*., 2007
Capitulatinoe cf. *cupisetis*	28S	KF919302	In this study
	18S	KF919301	In this study
	16S	KF919303	In this study
Ceratocephale loveni	28S	DQ442618	Ruta *et al*., 2007
	18S	DQ442616	Ruta *et al*., 2007
	16S	DQ442614	Ruta *et al*., 2007
Chaetoparia nilssoni	28S	AY996108	Eklof *et al*., 2007
	18S	AY996090	Eklof *et al*., 2007
	16S	AY996069	Eklof *et al*., 2007
Eunoe nodosa	28S	JN852854	Norlinder *et al*., 2012
	18S	JN852824	Norlinder *et al*., 2012
	16S	JN852892	Norlinder *et al*., 2012
E. cf. *oerstedi*	28S	KF006981	In this study
	18S	KF006979	In this study
	16S	KF006980	In this study
Eulalia mustela	28S	AY996105	Eklof *et al*., 2007
	18S	AY996086	Eklof *et al*., 2007
	16S	AY996065	Eklof *et al*., 2007
Gastrolepidia clavigera	28S	JN852855	Norlinder *et al*., 2012
	18S	JN852825	Norlinder *et al*., 2012
	16S	JN852893	Norlinder *et al*., 2012
Glycera tridactyla	28S	HM746739	Paul *et al*., 2007
	18S	HM746726	Paul *et al*., 2007
	16S	HM746711	Paul *et al*., 2007
Harmothoe glabra	28S	JN852858	Norlinder et al., 2012
	18S	JN852828	Norlinder *et al*., 2012
	16S	JN852896	Norlinder *et al*., 2012
H. impar	28S	JN852859	Norlinder *et al*., 2012
	18S	JN852829	Norlinder *et al*., 2012
	16S	JN852897	Norlinder *et al*., 2012
H. oculinarium	28S	JN852860	Norlinder *et al*., 2012
	18S	AY894299	Struck *et al*., 2005
	16S	JN852898	Norlinder *et al*., 2012
Leocrates chinensis	28S	DQ442605	Ruta *et al*., 2007
	18S	DQ442589	Ruta *et al*., 2007
	16S	DQ442575	Ruta *et al*., 2007
Lepidonotus clava	28S	JN852864	Norlinder *et al*., 2012
	18S	JN852833	Norlinder *et al*., 2012
	16S	JN852902	Norlinder *et al*., 2012
Neoleanira tetragona	28S	JN852872	Norlinder *et al*., 2012
	18S	AY839570	Wiklund *et al*., 2005
	16S	JN852911	Norlinder *et al*., 2012
Nereis pelagica	28S	AY340407	Rousset *et al*., 2007
	18S	AY340438	Rousset *et al*., 2007
	16S	AY340470	Rousset *et al*., 2007
Paradyte crinoidicola	28S	JN852869	Norlinder *et al*., 2012
	18S	JN852837	Norlinder *et al*., 2012
	16S	JN852907	Norlinder *et al*., 2012
Outgroup			
Liponema brevicornis	28S	EU190827	Daly *et al*., 2008
	18S	EU190866	Daly *et al*., 2008
	16S	EU190784	Daly *et al*., 2008

prostomium (Figure 2A). Parapodia are subbiramous with a larger neuropodium and smaller notopodium. The notopodium is unsharpened, digitiform, absent notosetae and positioned on anterodorsal side of neuropodium. Neuropodium is bluntly rounded with presetal lobe and bear rows of neurosetae on basal semilunar pocket. Aciculae embedded within notopodium and neuropodium throughout, proximally to distally. Notopodia and neuropodia bear dorsal and ventral cirri, respectively. Dorsal cirri are typically longer than ventral cirri (Figure 2B). Neurosetae in the half-moon-shaped blades, serrate, long, with conspicuously bidentate tips, wide subdistally, except neurosetae of first parapodia are short (Figures 2C and D). Elytra are orbicular, large, soft, pellucid and without tubercles (Figure 2E).

In *E.* cf. *oerstedi*, the body is oblong, with an average size of 10 mm long by 3.5 mm wide, covered by fifteen pairs of elytra (Figure 1C). Prostomium bilobed with two pairs of eyes, with cephalic peaks obviously, three antennae, paired palps, two paired tentacular cirri. Two pairs of eyes occur dorsolaterally on prostomium part. Median antenna is larger and longer than paired lateral antennae. Two pairs of tentaculophore are approximately cylindrical on anterolateral side of prostomium (Figure 2F). Paired elytrophores are cylindrical, short, wide plates on tips at dorsal setigers. Parapodia are biramous with a notopodium smaller than the neuropodium. Notopodium is rounded with short acicular lobe, and neuropodium is subconical with long acicular lobe. Aciculae embedded within notopodium and neuropodium project proximally to distally. Numerous well-developed setae on basal semilunar pocket of notopodium and neuropodium (Figure 2G). Notosetae are stout, long, thick, and with capillary tips adorned by rows of serrations. Neurosetae are similar to notosetae in aspect but are longer (Figures 2H and I). Elytra are reniform, light brownish, translucent, with macrotubercles on posterior margins (Figure 2J). Macrotubercles are digitiform, stout, variable in size and with prong branching on tips (Figure 2K).

3.2 SEM investigation of polynoid surfaces

In dorsal view of *C.* cf. *cupisetis*, the surface of prostomium is very bumpy, but the surfaces of antennae, palps, tentacular cirrus, dorsal cirrus and parapodia are slightly rough. Anterior parts of elytra are rougher than the posterior parts (Figure 3A). In ventral view, the surface of buccal cirri is as rough as the surface of antennae and other regions. The surface with little roughness covers the ventral body, including around the mouth. However, the surface is very bumpy on the anteromedial side (Figure 3B). Neurosetae of the first parapodia are smooth with rows of serration. One row of serration on sub-distal setae is broad and surrounds the edge with multiple saw-teeth. Four paired serrations on notched tip of setae show the digitiform row of eleven saw-teeth (Figures 3C and D). Surface of neurosetae on the second parapodia and others is similar to the first parapodia. However, there are differences in the number of serrations. The second parapodia and others consist of six or seven paired serrations with six to eleven saw-teeth (Figures 3E and F).

In dorsal view the surface of *E.* cf. *oerstedi* is irregular, wrinkled and supported by transverse gracile folds. Narrow groove lines run longitudinally along the mediodorsal body. Tentaculophore and cirrophore surfaces are slightly rough. Plate surfaces on tips of elytrophores are approximately smooth. Filament-like structures are present on surfaces of tentacular cirri. The prostomium surface is bumpy. Two pairs of anterior dorsal cirri present a seta-like structure (Figure 4A). Anterior dorsal cirri are stout, smooth, with blunt tips and bear rows of serrations without saw-teeth on edges. Three antennae are hidden by a cluster of filament-like structures. A high degree of corrugation is found on palp surfaces with short filaments (Figure 4B). Cirrophores and pygidium are moderately rough, and posterodorsal surfaces are wrinkled. Narrow groove line is not connected on posterodorsal surface (Figure 4C). In ventral view, the surface of the mouth opening is slightly wrinkled. Buccal cirri are also bumpy (Figure 4D). Notosetae and neurosetae with smooth surfaces are surrounded by serrations approximately three-fourths the width of setae. Each serration presents crenulations on margin (Figures 4E and F). The anterior and middle areas of the elytra are covered with numerous coin-shaped microtubercles. In contrast, no microtubercles or macrotubercles are observed on proximo-lateral part. The posterior areas bear scattered macrotubercles and are covered by a meshwork of microfilaments on surfaces. Macrotubercles are adorned with small papillae and occur trifid to pentafid on tips (Figure 4H).

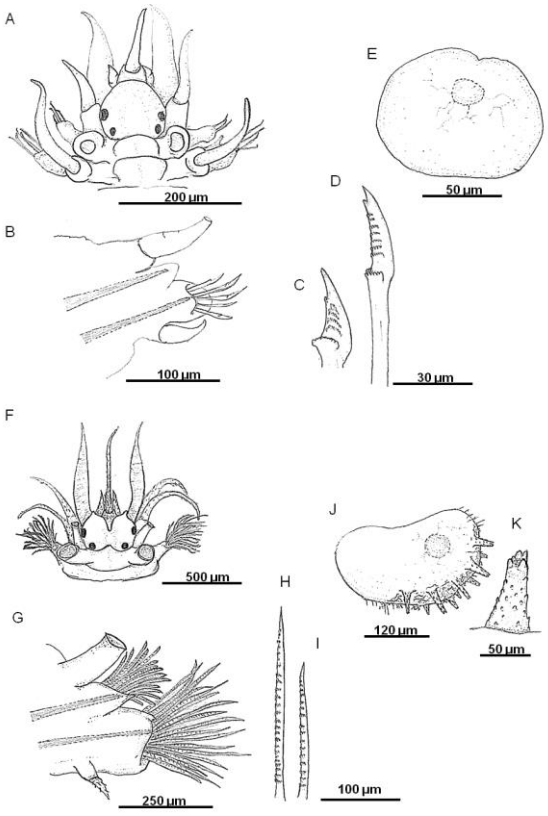

Figure 2. General morphology of *C.* cf. *cupisetis*: anterior part (A), parapodium of middle segment (B), neurosetae of first parapodium (C), neurosetae of middle parapodium (D), elytrum of middle segment (E). General morphology of *E.* cf. *oerstedi*: anterior part (F); parapodium of middle segment (G), notosetae of middle parapodium (H), neurosetae of middle parapodium (I), elytrum of middle segment (J), macrotubercle (K).

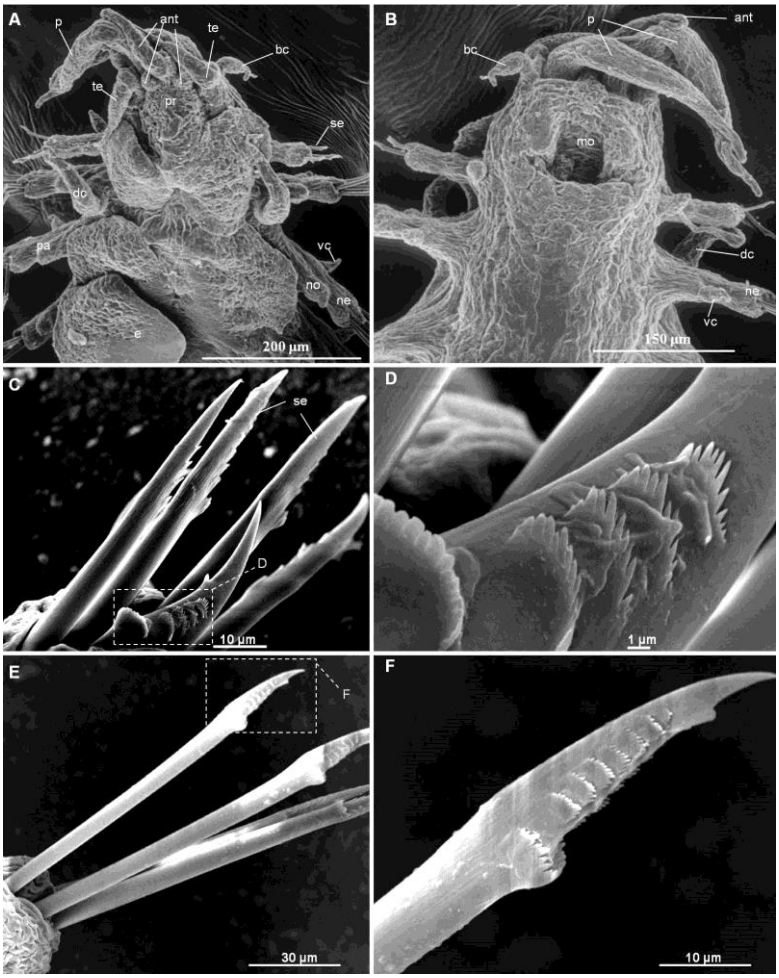

Figure 3. Surface topography of *C.* cf. *cupisetis*: dorsal view of anterior part (A), ventral view of anterior part (B), neurosetae of first parapodium (C), a higher magnification of outlined area in Figure C (D), neurosetae of middle parapodium (E), a higher magnification of outlined area in Figure E (F). ant, anterior part; bc, buccal cirrus; dc, dorsal cirrus; e, elytrum; mo, mouth; ne, neuropodium; no, notopodium; p, palp; pr, prostomium; se, setae; te, tentacular cirrus; vc, ventral cirrus.

3.3 Molecular phylogenetic analysis of rDNA genes

The phylogenetic relationships of *C.* cf. *cupisetis* and *E.* cf. *oerstedi* with eighteen other polychaete species and one cnidarian was analysed using the 28S, 18S and 16S rDNA genes. Results indicated that species belonging to the Polynoidae and Sigalionidae share recent common ancestry but that those of the Phyllodocidae, Hesionidae, Glycerdae and Nereididae do not. *Capitulatinoe* cf. *cupisetis* and *E.* cf. *oerstedi* were included the Polynoidae which was the sister group to the Sigalionidae (*Neoleantra tetragona*). In the polynoid subfamily Arctonoinae, *C.* cf. *cupisetis* was closely related to *Gastrolepidia clavigera* and was the sister taxon to *Paradyte crinoidicola*. In the Polynoinae, *E.* cf. *oerstedi* was the sister taxon to *H. impar* and the group containing *H. oculinarium*, *H. glabra* and *E. nodosa*. Both subfamilies together formed the sister group of the Lepidonotinae (*Lepidonotus clava*). The rDNA sequences clearly distinguished polynoid species from each of the two species of Hesionidae (*Leocrates chinensis* and *Amphiduros fuscescens*), Phyllodocidae (*Chaetoparia nilssoni* and *Eulalia mustela*), and Nereididae (*Ceratocephale loveni* and *Nereis pelagica*), and the one species of Glycerdae (*Glycera tridactyla*). All worms were distantly related to the outgroup (Cnidaria: *Liponema brevicornis*) (Figure 5).

4. Discussion

For this study comparison were made with specimen materials and other data. Based on *C.* cf. *cupisetis* specimen found in the ambulacral grooves of the starfish *A. indica* from the Gulf of Thailand, we described the general morphology of adults of the commensal *C.* cf. *cupisetis*. Similarly of *C. cupisetis* was originally found in the ambulacral grooves of the common starfish *A. granulatus* in Broome, Western Australia (Hanley & Burke, 1989) and was used to describe the detailed morphology of these scale-worms. The specimens in Thailand resemble the Australian *C. cupisetis*: a similar structure was found in the antennae, palps, prostomium, cirri, setigers, parapodia, saetae and elytra. In addition, we found that is *C.* cf. *cupisetis* a commensal species of *A. indica*.

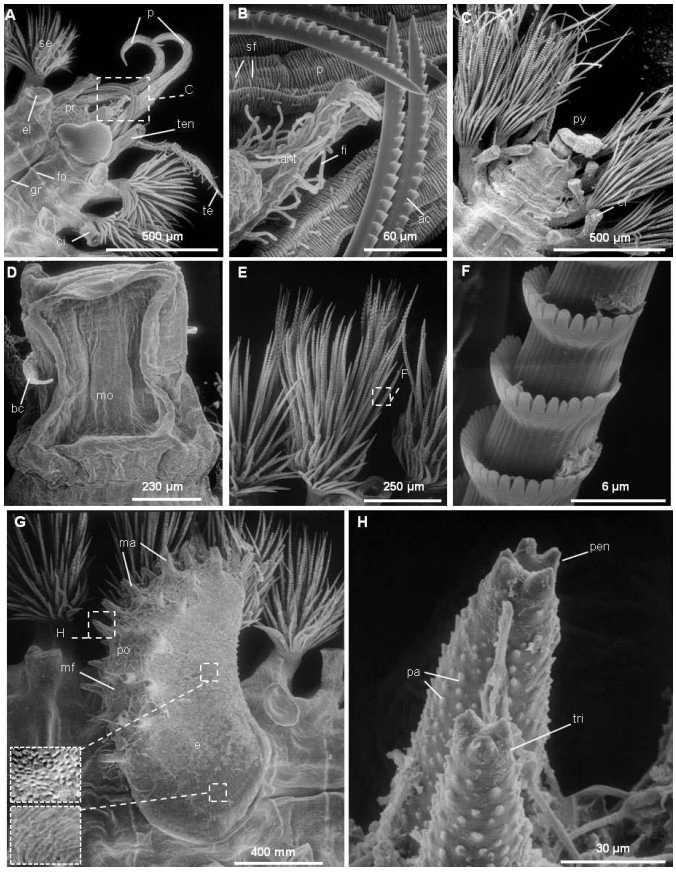

Figure 4. Surface topography of *E.* cf. *oerstedi*; dorsal view of anterior part (A), a higher magnification of outlined area in Figure A (B), ventral view of anterior part (D), neurosetae of middle parapodium (E), a higher magnification of outlined area in Figure E (F), elytrum of middle segment (G), macrotubercle in a higher magnification of outlined area in Figure G (H). ant, anterior part; ac, anterior cirrus; bc, buccal cirrus; ci, cirrophore; el, elytrophore; dc, dorsal cirrus; e, elytrum; fi, filament; fo, fold; gr, groove; ma, macrotubercle; mi, microfilament; mo, mouth; ne, neuropodium; no, notopodium; p, palp; pa, papillae; pen, pentafid tip; po, posterior part; pr, prostomium; py, pygidium; se, setae; sf, short filament; te, tentacular cirrus; ten, tentaculophore; tri, trifid; vc, ventral cirrus.

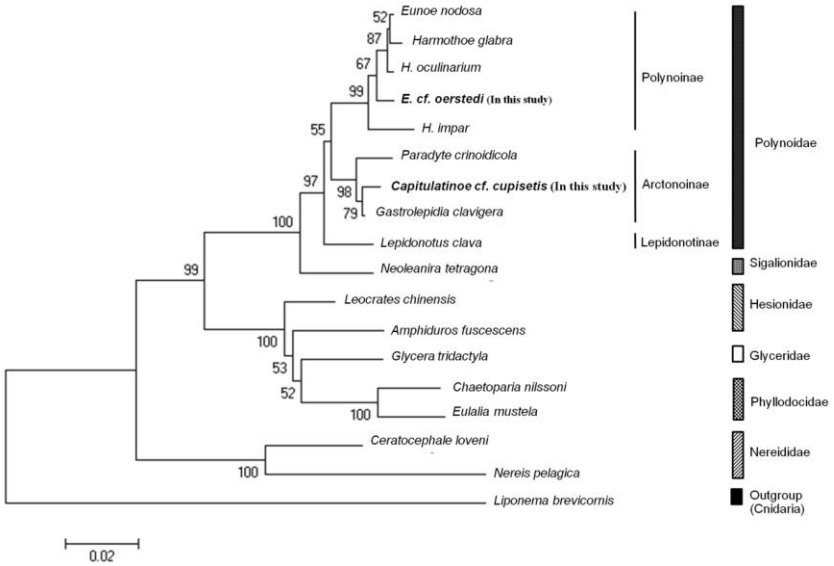

Figure 5. Neighbor-joining tree with 1000 replications for selected polychaetes showing the relationships of *C.* cf. *cupisetis* and *E.* cf. *oerstedi* with fifteen polychaetes and one outgroup species using partial nucleotide sequences of 28S, 18S and 16S rDNA.

For *E.* cf. *oerstedi*, our morphological description corresponds with that of Banse and Hobson (1974), in which *E. oerstedi*, with the unidentate character of its setae tips, was distinguished from the fifteen-scaled worm *Harmothoe*. The elytra of our specimens are similar to those described previously by Pollock (1998) from specimens of eastern North America, with branched macrotubercles on the elytrum surfaces. However, the worms in the Gulf of Thailand seem to be shorter than those in eastern North America.

The surface morphology of *C. cupisetis* and *E. oerstedi* has been examined the present study by SEM. The descriptions of the surfaces of the saetae and elytra are consistent with observations made under stereomicroscopy by Hanley and Burke (1989) of *C. cupisetis* and by Malmgren (1866) and subsequent authors of *E. oerstedi*. In the present study, the results of SEM provided detail of the worm surfaces beyond those available from microscopic observations, including details of the epidermal, seta and elytrum surfaces in *C.* cf. *cupisetis* and of the cirri, pulp, saetae, microtubercles and macrotubercles in *E.* cf. *oerstedi*.

Based on the 28, 18S and 16S rDNA analyses, *C.* cf. *cupisetis* and *E.* cf. *oerstedi* were recovered in the Arctonoinae and Polynoinae, respectively, as expected a sister group to the Lepidonotinae. These molecular data identified the Arcto-noinae and Polynoinae as monophyletic within the Poly-noidae. These results are also in agreement with Norlinder *et al*. (2010), which classified the Polynoidae as monophyletic based on the nuclear and mitochondrial sequences of 48 taxa of scale-worm polychaetes. Within the Arctonoinae, the clade topology of the present study identifies *C.* cf. *cupisetis* as more closely related to *G. clavigera* than to *P. crinoidicola*. This result appears congruent with SEM observations of setae morphology of *G. clavigera* and *P. crinoidicola* by Britayev *et al*. (1999): neurosetae stout with subdistal swelling; rows of fine serrations present on the neurosetae of *G. clavigera* but not *P. crinoidicola*. Therefore, the appearance of serrations may be in accordance with the molecular relationships of these species. In the Polynoinae, *E.* cf. *oerstedi* was not monophyletic with *E. nodosa* within the Polynoidae in the present study. However, to date, the elytrum tubercles and other morphological characters of the latter species have been regarded as incongruent with the molecular relationships of this subfamily.

To our knowledge, the present study provides the report of *C.* cf. *cupisetis* and *E.* cf. *oerstedi* in the Gulf of Thailand and provides morphological and molecular information on both scale polychaetes; however, comparison with real specimens, additional and supporting data are needed for their precise identification as *C. cupisetis*, *E. oerstedi* or cryptic species.

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