



Faculty of Resource Science and Technology

**Morphological Characterization of Polychaete: Phylogenetic Insight into
Eulepethids from Buntal Beach, Sarawak**

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Morphological Characterization of Polychaete: Phylogenetic Insight into
Eulepethids from Buntal Beach, Sarawak

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DECLARATION

I declare that the work in this thesis was carried out in accordance with the regulations of Universiti Malaysia Sarawak. Except where due acknowledgements have been made, the work is that of the author alone. The thesis has not been accepted for any degree and is not concurrently submitted in candidature of any other degree.



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ABSTRACT

Globally, over 10,000 polychaete species inhabit marine environment, from intertidal to deep ocean areas. In Malaysia, the taxonomic knowledge of this specific class remains inadequate, with a notable scarcity of comprehensive understanding. Moreover, an abundance of species within this class presents classification raises uncertainties or is under debate. Despite the longstanding documentation of 57 polychaete species across 30 families in Peninsular Malaysia since the early 1870s, Sabah and Sarawak have recorded only 11 species across 7 families. This project encompassed of morphological identification of all samples, and molecular analysis for the dominant species found in intertidal zone of Buntal Beach, Sarawak. The first part integrated conventional identification method and SEM analysis, which led to the identification of 10 polychaete species. The identified species are (1) *Buntalepis sarawakensis* **sp. nov.** from Family Eulepethidae; (2) *Glycera tridactyla*, (3) *Glycera alba* and (4) *Glycera tessellata* from Family Glyceridae; (5) *Micronephthys sphaerocirrata* from Family Nephtyidae; (6) *Onuphis eremita*, (7) *Onuphis* sp. 1 and (8) *Onuphis* sp. 2 from Family Onuphidae; (9) *Euchone* sp. 1 from Family Sabellidae; (10) *Scoelepis (scoelepis) squamata* from Family Spionidae;. With a total of 116 individuals collected, the Eulepethidae species were found dominant, representing 22% of the samples, while the Spionidae species was the least abundant, comprising only 3%. Morphological analysis of Eulepethidae species revealed distinct characteristics, including a fixed position of the starting posterior lamellae and the presence of unique middle neurochaetae, differentiating them from other genera and species within the same family. Therefore, molecular analysis was conducted for the Eulepethidae species to further determine its evolutionary relationships and resolve taxonomic uncertainties. BLAST results for

mitochondrial COI, 16S, and ribosomal 28S sequences of the Eulepethidae species revealed less than 85% similarity to its closest relatives in the Eulepethidae family. Phylogenetic analysis using MP, ML, and BI trees showed that the six isolates belonged to the Eulepethidae family but formed a well-supported monophyletic clade (100% bootstrap), distinct from the genera *Eulepethus*, *Grubeulepis* and *Mexieulepis*. Intriguingly, this study unveiled the identification of a new genus. This study documented 10 species with detailed identification and molecular data of a new genus, *Buntalepis* **gen. nov.**, and its species, *B. sarawakensis* **sp. nov.**, addressing scientific gaps in polychaete diversity, new species discovery, integration of conventional and molecular techniques, and insights into phylogenetic relationships, specifically at Buntal Beach, Sarawak.

Keywords: 16S, 28S, Buntal beach, COI, morphological identification

Taksonomi polychaeta (Annelida: Polychaeta) dan Analisa Filogenetik Eulepethids dari Pantai Buntal, Sarawak.

ABSTRAK

Secara global, lebih daripada 10,000 spesies polychaete mendiami persekitaran marin, dari kawasan pasang surut hingga ke kawasan laut dalam. Di Malaysia, pengetahuan taksonomi kelas khusus ini masih tidak mencukupi, dengan kekurangan yang ketara dalam pemahaman yang komprehensif. Selain itu, banyaknya spesies dalam kelas ini menimbulkan ketidakpastian atau perdebatan tentang pengkelasan. Walaupun telah didokumenkan sebanyak 57 spesies polychaete merentasi 30 keluarga di Semenanjung Malaysia sejak awal tahun 1870-an, Sabah dan Sarawak hanya mencatatkan 11 spesies merentasi 7 keluarga. Projek ini merangkumi pengenalan morfologi semua sampel, dan analisis molekul untuk spesies dominan yang terdapat di zon intertidal Pantai Buntal, Sarawak. Bahagian pertama mengintegrasikan kaedah pengenalan konvensional dan analisis SEM, yang membawa kepada pengenalan 10 spesies polychaete. Spesies terkumpul adalah (1) Buntalepis sarawakensis **sp. nov.** daripada Keluarga Eulepethidae; (2) Glycera tridactyla, (3) Glycera alba dan (4) Glycera tessellata daripada Keluarga Glyceridae; (5) Micronephthys sphaerocirrata daripada Keluarga Nephtyidae; (6) Onuphis eremita, (7) Onuphis sp. 1 dan (8) Onuphis sp. 2 daripada Keluarga Onuphidae; (10) Euchone sp. 1 daripada Keluarga Sabellidae; (9) Scoelepis (scoelepis) squamata daripada Keluarga Spionidae. Dengan jumlah keseluruhan 116 individu yang dikumpul, spesies Eulepethidae didapati dominan, mewakili 22% daripada sampel, manakala spesies Spionidae adalah paling kurang banyak, hanya 3%. Analisis morfologi spesies Eulepethidae mendedahkan ciri-ciri yang berbeza, termasuk kedudukan tetap lamella posterior permulaan dan kehadiran neurochaetae tengah yang unik, membezakannya daripada genus dan spesies lain dalam keluarga yang sama.

Oleh itu, analisis molekul telah dijalankan untuk spesies Eulepethidae untuk menentukan hubungan evolusi dan menyelesaikan ketidakpastian taksonomi. Keputusan BLAST untuk urutan COI, 16S, dan ribosomal 28S mitokondria bagi spesies Eulepethidae menunjukkan persamaan kurang daripada 85% dengan saudara terdekatnya dalam keluarga Eulepethidae. Analisis filogenetik menggunakan pokok MP, ML, dan BI menunjukkan bahawa enam isolat tergolong dalam keluarga Eulepethidae tetapi membentuk klad monofiletik yang disokong dengan baik (100% bootstrap), berbeza daripada genus Eulepethus, Grubeulepis dan Mexieulepis. Menariknya, kajian ini mendedahkan pengenalan genus baharu. Kajian ini mendokumenkan 10 spesies dengan pengenalan terperinci dan data molekul bagi genus baharu, Buntalepis **gen. nov.**, dan spesiesnya, B. sarawakensis **sp. nov.**, menangani jurang saintifik dalam kepelbagaian polychaete, penemuan spesies baharu, integrasi teknik konvensional dan molekul, dan pandangan tentang hubungan filogenetik, khususnya di Pantai Buntal, Sarawak.

Kata kunci: 16S, 28S, Pantai Buntal, COI, pengelasan morfologi

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LIST OF ABBREVIATIONS

°C	Degree Celcius (temperature)
µl	Microliter
µM	Micromolar
AGE	Agarose Gel Electrophoresis
BLAST	The Basic Local Alignment Search Tool
bp	Base pair
COI	Cytochrome Oxidase I
CTAB	Cetyltrimethylammonium Bromide
DNA	Deoxyribonucleic Acid
dNTP	Deoxyribonucleic triphosphate
ddH ₂ O	Deionized distilled water
EtBr	Ethidium bromide
MEGA	Molecular Evolutionary Genetics Analysis
MgCl ₂	Magnesium chloride
ML	Maximum Likelihood
mm	Millimeter
mM	Millimolar
MP	Maximum Parsimony
mtDNA	Mitochondrial Deoxyribonucleic acid
n	Number of samples
ng	Nanogram
NJ	Neighbour-Joining

PAUP	Phylogenetic Analysis using Parsimony
PCR	Polymerase Chain Reaction
TAE	Tris-acetate-ethylenediaminetetraacetic
UV	Ultraviolet
V	Volt

CHAPTER 1

GENERAL INTRODUCTION

1.1 Polychaete and its Background Study

Polychaetes, or bristle worms, belong to the class Polychaeta within the phylum Annelida. Polychaetes are predominantly marine organisms, although some species inhabit freshwater and brackish environments. Polychaete is a small invertebrate marine organism, though some species can grow to be 6 meters long (Rouse & Pleijel, 2001). Compared to other taxa of similar ecological significance, polychaetes remain largely understudied (Quijon & Snelgrove, 2005) as many researchers consider them to be less important due to their intermediate positions in the food web (primary or secondary users) (Jumars *et al.*, 2015). However, polychaetes play a vital role in ecosystem processes such as pollutant metabolism, nutrient cycling, dispersion, and burial and also as secondary production (Abdullah & Lee, 2017). According to Prabakaran *et al.* (2019), polychaetes, which are the most prevalent groups in benthic infaunal communities, make up around 80% of the entire macrobenthic population and consume microbial (bacteria, microalgae, protists, and fungus) as well as meiobial and organic substances. Polychaetes are integral to the trophic dynamics of many ecosystems, serving as primary prey for a range of ecologically and conservationally significant species, including fish, birds, and mammals (Hutchings, 1998; Jumars *et al.*, 2015; Martins & Barros, 2022). Polychaetes contribute substantially to ecosystem functions such as nutrient cycling and ecosystem engineering through activities like bioturbation and bio-irrigation (Kristensen *et al.*, 1985). Furthermore, certain polychaete species are economically significant in several European countries as these species are harvested for use as bait (Gambi *et al.*, 1994; Olive, 1994; Gillet & Torresani, 2003).

Conventional morphological taxonomy has been foundational for species identification for over a century, focusing on key aspects like external morphology (Buhre & Purchke, 2021), reproductive structures (Hutchings, 2000), tube structure (Fauchald, 1977), and cuticular structures (Ferri *et al.*, 2024) in polychaetes. For the past 250 years, taxonomy has relied on morphological structures and the phenotypic characteristics of individual organisms (Hebert & Gregory, 2005). Comparative anatomy has generated a diverse terminology to describe the differences among species (Howe, 1960; Young, 1989; Park, 2008; Winfield *et al.*, 2009; Bijl & Brinkhuis, 2015; Harvey, 2015; Matalin, 2015). These macromorphological features allow for visual differentiation among species. However, conventional morphological identifications of polychaetes are often confused with other benthic animals such as Nemertea, Echiura, Priapulida, Phoronida and Sipuncula. For example, the peristomial segments are often difficult to identify (Pattaratumrong & Wongkhamhaeng, 2019). The limitations associated with this method, such as the difficulty in distinguishing between similar-looking species and the potential for misidentification, highlight the need for complementary techniques, such as DNA analysis.

Molecular methods, such as PCR and DNA sequencing, offer a more accurate and reliable approach for species delimitation and understanding biodiversity (Wu *et al.*, 2023). Among the most commonly used molecular markers for polychaetes are the mitochondrial COI gene, 16S rRNA gene, and 28S rRNA gene (Canales-Aguirre *et al.*, 2011; Wu *et al.*, 2022; Chen *et al.*, 2021; Castro Álvarez *et al.*, 2023; Lim *et al.*, 2023; Selifanova *et al.*, 2023). These genes have been successfully employed in various studies to identify species, reconstruct phylogenies, and investigate population genetics and biogeography. For instance, Zhou *et al.* (2010) utilized COI and 16S rRNA to identify polychaete species and reconstruct their phylogenetic relationships, while Guggolz *et al.* (2020) used 16S rRNA to

identify distinct lineages of *Prionospio* and *Aurospio*. Additionally, studies by Eklöf *et al.* (2007) and Kupriyanova *et al.* (2023) have employed 28S rRNA to examine population structure and phylogeography of polychaete species. However, it's essential to consider the limitations of each marker and to combine them with morphological data for comprehensive species identification and analysis.

Early studies, such as Rullier's (1976) in the 1990s, identified new polychaete species in Malaysian waters. Subsequent research has led to the discovery of many new species (Idris & Arshad, 2013; Idris *et al.*, 2014; Ibrahim *et al.*, 2019). However, the documentation of polychaetes in Malaysia, particularly Sarawak is still notably lacking, despite their diversity in the marine environment (Glasby *et al.*, 2000). Buntal Beach in Sarawak, Malaysia, presents an ideal location for polychaete research due to its ecological significance and diverse environments. As part of a critical fishery zone, Buntal Beach supports a wide range of benthic organisms, including economically valuable species such as *Solen* spp. (Siti, 2011). The intertidal zone of Buntal Beach extends for 2 km and features a variety of habitats, including mudflats, sandy shores, and mangroves, which are known to support diverse polychaete species. However, research on their biodiversity and ecological roles has been limited. Previous study (Zakirah *et al.*, 2019) have identified several polychaete species in the region, but the genetic diversity and precise taxonomic classification of these species remain largely unexamined. Therefore, further research is essential for improving species identification and conservation in Sarawak.

1.2 Problem Statement

Taxonomy and systematics are crucial for understanding biodiversity, traditionally relying on external morphological descriptions. This method, while widely used in

polychaete taxonomy, presents significant challenges, particularly regarding morphologically identical species. The process is complex, requiring detailed characterization and expert identification, which is time-consuming and often restricted by the need for extensive experience. As a result, new or unknown species may be overlooked or misidentified, compromising biodiversity assessments (Ko *et al.*, 2013). For example, early studies on *Perinereis* species initially suggested that *Perinereis gualpensis* and *Perinereis vallata* could be a single species based solely on morphological comparisons. Subsequent research revealed sufficient morphological and ecological differences to classify them as distinct species (Bertrán, 1980), which was later validated by phylogenetic analyses integrating both molecular and morphological data (Sampertegui *et al.*, 2013). These limitations in conventional biodiversity assessment methods underscore the necessity for emerging approaches in species identification.

In Malaysia, particularly in Borneo—one of the world’s biodiversity hotspots (Myers *et al.*, 2000)—there is a notable lack of genetic studies on polychaete species. The dominant species along Sarawak’s coast remain unconfirmed genetically, resulting in significant gaps in knowledge regarding their diversity and distribution. This study posits that relying solely on conventional morphological methods is insufficient for accurately classifying polychaetes at the genus or species level, particularly for morphologically similar species. Thus, integrating morphological and molecular data is essential to overcome the limitations of traditional taxonomy and ensure accurate classification based on phylogenetic relationships. Ultimately, molecular methods can enhance morphological identification and provide valuable genetic insights, leading to a more comprehensive understanding of species diversity, relationships, and distinctions at both species and individual levels.

1.3 Objectives

The objectives of this study were:

- i. To identify the general polychaete distribution in Buntal beach by conventional morphological identification, provide dichotomous keys and taxonomical drawing of collected species for detailed morphological comparison.
- ii. To construct the phylogenetic tree of the dominant polychaete species found in Buntal beach by using mitochondrial DNA which are Cytochrome Oxidase Subunit-1 (COI) and Ribosomal DNA, 16S and 28S rRNA sequences.

CHAPTER 2

LITERATURE REVIEW

2.1 Polychaete Taxonomy

Polychaetes, the largest class within the phylum Annelida, have a rich history of study. The first cladistic analysis, encompassing 53 families, was conducted by Rouse and Fauchald in 1997. As of 2024, the World Register of Marine Species (WoRMS) recognizes over 11,456 valid species, distributed across approximately 87 families and 1,200 genera. This ongoing discovery highlights the dynamism and complexity of polychaete diversity. Despite their morphological diversity, polychaetes share a common body structure (Westheide & Rieger, 1996; Fauchald & Rouse, 1997; Blake & Arnofsky, 1999; Costello, 2001; Ditlev, 2003; Purschke, 2005). This structure is rooted in their developmental stages. The prostomium, pygidium, and trunk each originate from specific regions of the trochophore, a larval form characteristic of many marine invertebrates (Brusca & Brusca, 2003). Generally, the polychaete body consist of three essential sections: the posterior region (pygidium), the trunk with various segments, and the anterior region or head (prostomium and peristomium). Each part originates from a distinct section of the trochophore. The prostomium develops from the pretrochal region located in front of the ciliated girdle (prototroch), the pygidium forms from the posterior end that bears the telotroch, and the metastomium results from the segmental division between the prototroch and telotroch.

2.1.1 Prostomium

The prostomium, a pre-oral lobe, contains the cerebral ganglia and the primary sense organs (Rouse & Fauchald, 1997). In primitive forms, it includes two pairs of eyes, three

antennae (one median and two lateral), a pair of ventro-lateral palps, and a pair of postero-lateral nuchal organs in the form of ciliated pits or grooves (Bartolomaeus & Purschke, 2002). In certain families, such as Nereididae, the distal part of the palp (palpostyle) is separated from the proximal part (palpophore) by a deep groove, resulting in a two-jointed structure (Bleidorn & Vogt, 2005). The prostomial sense organs are most highly developed in Polychaeta Errantia, which are typically active carnivores (Rouse & Fauchald, 1997). In Polychaeta Sedentaria, which are inactive microphagous feeders, the antennae, prostomial palps, and even the eyes may be absent, resulting in the prostomium fusing and becoming indistinguishable from the first metastomium (Aronson & Gilbert, 2019). Additionally, in errantial forms that have adopted burrowing habits, many peristomial segments or the peristome develop specialized food-gathering organs (Rouse & Fauchald, 1997).

The mouth is located on the peristomium, the segment behind the prostomium. Before the mouth, dorsally, is the prostomium, which may have eyes or appendages like palps, cirri, tentacles, or radioles (Bartolomaeus & Purschke, 2002). The term prostomium originates from the Greek words "*prostomion*" (mouth and lips), and "*pro-*" meaning in front (Hutchings, 2000). The mouth is ventrally supported by the peristomium, situated behind the prostomium (Rouse & Fauchald, 1997). A polychaete's mouth is situated on the ventral portion of the body, with the dorsal portion opposite (Bartolomaeus & Purschke, 2002). These sensory structures can be found on free-living polychaetes that move by crawling, swimming, or burrowing with parapodia (Rouse & Fauchald, 1997). Meanwhile, these sensory structures are absent in sedentary polychaetes (Bartolomaeus & Purschke, 2002).

The presence of the jaw and pharynx depends on and adapts to the feeding habit and diets of polychaetes (Bleidorn & Vogt, 2005). In some species, the pharynx is modified into

a long proboscis (Bartolomaeus & Purschke, 2002). Polychaetes have demonstrated remarkable evolutionary adaptability, conquering diverse habitats ranging from marine depths to terrestrial environments. These segmented worms have evolved specialized adaptations to thrive in various conditions. Some species have developed cutaneous invaginations for aerial gas exchange, enabling them to survive in terrestrial environments with limited access to water (Martin & Glasby, 2006). Others have adapted to freshwater habitats, tolerating low salinity, reduced oxygen levels, and varying water temperatures (Rouse & Fauchald, 1997). Most polychaetes burrow in the sediment, with some living as commensals and others as parasites (Rouse & Fauchald, 1997). Therefore, some polychaetes may have specialized gills or tentacles used for respiration and deposit or filter feeding (Bleidorn & Vogt, 2005). These evolutionary innovations highlight the versatility and resilience of polychaetes, showcasing their ability to thrive in a wide range of ecological niches.

2.1.2 Parapodia

The polychaete body comprises variously sized segments, each featuring paired lateral outgrowths known as parapodia (Adkins *et al.*, 2023). The term "parapodia" is derived from the Greek words "*para*," meaning beyond or besides, and "*podia*," meaning feet. These parapodia are essentially biramous, consisting of two branches: the ventral branch, known as neuropodia, and the dorsal branch, known as notopodia (Rouse & Pleijel, 2001; Adkins *et al.*, 2023). In certain families, parapodia may have only one branch, referred to as uniramous. Each parapodium typically includes chitinous bristles called chaetae, derived from the Greek word "*chaite*," meaning long hair (Adkins *et al.*, 2023). The dorsal chaetae are termed notochaetae, while the ventral ones are called neurochaetae. However, the presence of these bristles may vary among species. Chaetal shape and organization are key

characteristics used to identify polychaetes, reflecting the enormous diversity of arrangement and function exhibited by parapodia (Adkins *et al.*, 2023). Parapodia serve various purposes such as locomotion, gaseous exchange, protection, attachment, and controlling water flow within a tube, and they may be reduced or lost altogether depending on the species and habitat (Adkins *et al.*, 2023).

2.1.3 Pygidium

The terminal body part encompassing the terminal anus is referred to as a pygidium, derived from the Greek word "*pugidion*," a diminutive of "*puge*," meaning buttocks (Fauchald, 1977). The pygidium constitutes the posterior portion of an annelid, and annelid grows by adding segments to the front of pygidium (Wilson, 2000). Polychaetes possess simple exit ducts from the gonads and pygidium, which serves as the tail housing the anus (Brusca & Brusca, 2003). Figure 2.1 showed example of a full body overview of Nereididae and Capitellidae family, with a shared commonality in their overall body composition.

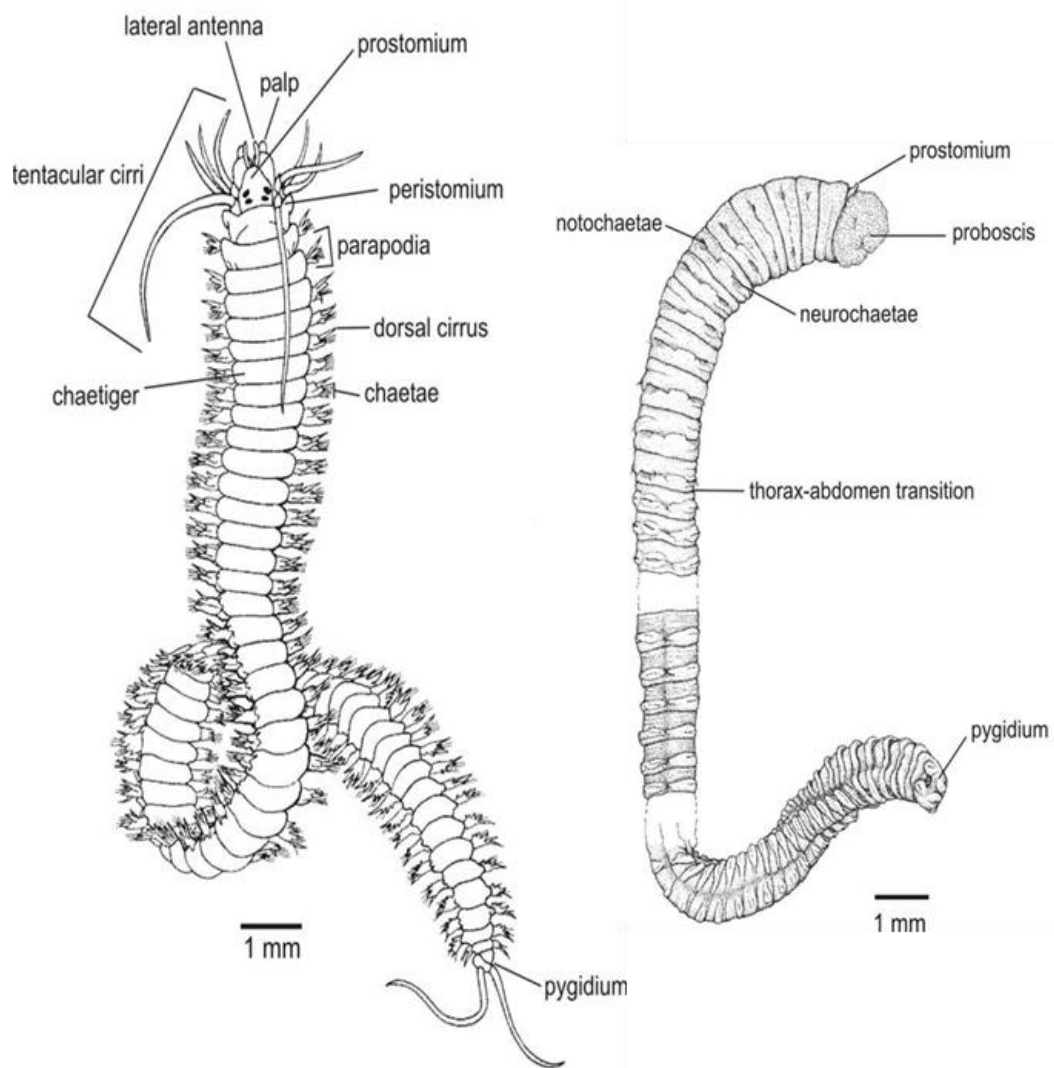


Figure 2.1: Full body overview of a polychaete belonging to the Nereididae family (left) and *Notomastus torquatus* (right) belonging to the family Capitellidae (Loo *et al.*, 2006).

2.2 Characteristics of Studied Family

2.2.1 Family Eulepethidae Chamberlin, 1919

The Eulepetids are a relatively small family, currently with about 24 species in the world and about six genera recorded under this family which are *Eulepethus*, *Grubeulepis*, *Lamelleulepethus*, *Mexieulepis*, *Pareulepis*, and *Proeulepethus* (Woolley & Wilson, 2011, Zhang *et al.*, 2017). Eulepetids inhabit tropical waters, in intertidal, subtidal depths between 400 and 800 m (Rouse & Pleijel, 2001, Woolley & Wilson, 2011). Most of the species are free-living, burrowing, and reside in sand, clay and mud bottoms (Pettibone, 1969); however, others have been reported associated with other scale worms or with tubes of empty chaetopterids (Nishi, 2001). The family is identified by its distally-expanded neuroacicula in the form of a "hammer head". Eulepetids are distinguished from other scale worms by their elytra-covered backs and unique leg arrangement. Only the 3rd and 6th legs possess dorsal cirri, while the remaining legs alternate with branchiae starting from segment 8 and continuing posteriorly. Figure 2.2 shows an overview of *Grubeulepis malayensis* from Eulepethidae family.

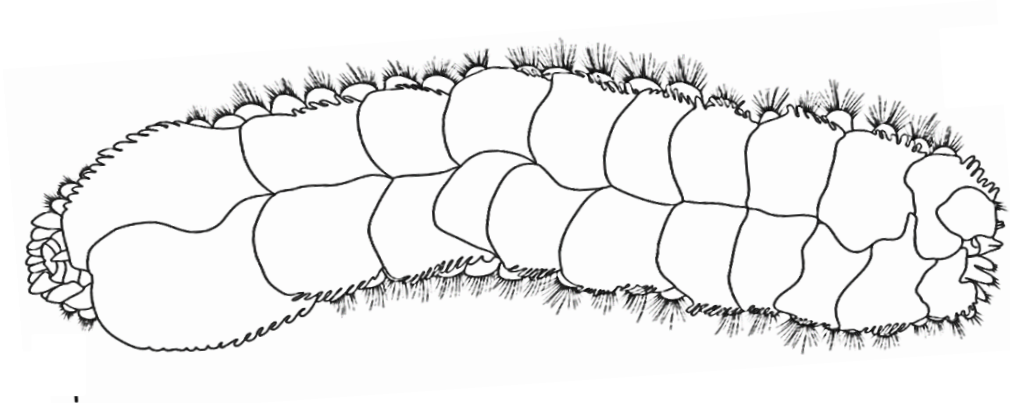


Figure 2.2: Example of Eulepethidae (*Grubeulepis malayensis* from Nishi, 2001)

2.2.2 Family Glyceridae Grube, 1850

The Glyceridae family is characterized by a unique feature: an exceptionally long proboscis that can extend over a considerable distance for feeding or burrowing purposes (Fauchald & Jumars, 1979). This distinctive trait serves as a valuable taxonomic characteristic that sets this family apart from other polychaete families. The surface of the proboscis features structures known as "proboscidial papillae," which are commonly used to identify members of this family taxonomically (Pettibone, 1963). These papillae are soft and lightly chitinized, typically tubular in shape, and may be arranged in a distinct longitudinal row encircling the proboscis, terminating in a terminal pore (Day, 1967). The papillae can vary in shape from broad and squat to long and conical, with smooth or ridged sides, transversely ringed, or featuring a striated flange at the distal end resembling a projecting fingernail (Purschke, 2005). In some genera of this family, the presence of jaw elements on the distal end of the proboscis is not uncommon (Day, 1967).

Glycerids are adept burrowers in sandy substrates, utilizing their eversible proboscis to excavate burrows and feed on prey using well-developed jaws located at the proboscis' end (Fauchald & Jumars, 1979). These jaws typically maintain a consistent shape, often black and heavily chitinized. Figure 2.3 provides an overview of *Glycera alba* from the Glyceridae family (Fox & Ruppert, 2006).

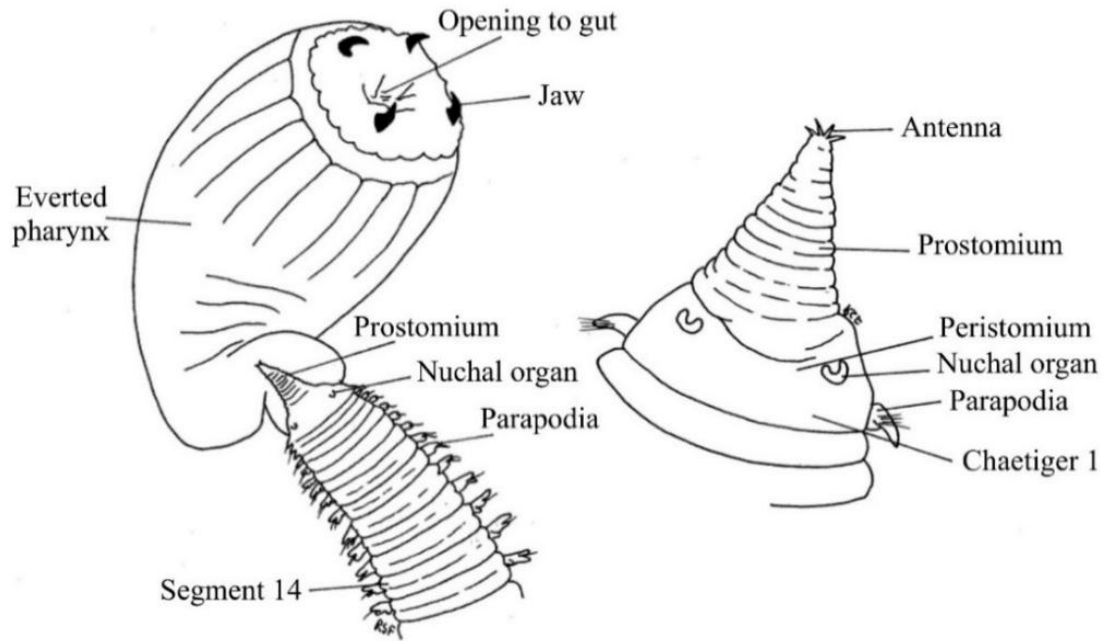


Figure 2.3: Example of Glyceridae (*Glycera alba* from Fox & Ruppert, 2006).

2.2.3 Family Nephtyidae Grube, 1850

Nephtyidae, commonly referred to as active burrowers, are frequently found inhabiting sandy mud areas, but they can also be found in other environments, such as coarser sediments or rocky bottoms (Hartman, 1950; Fauchald, 1977). Their adaptability to different substrates is likely due to their burrowing abilities and their ability to tolerate varying oxygen levels and food availability. Employing their eversible proboscis, these organisms exhibit remarkable agility in actively burrowing through various grades of sand and sandy mud substrates. Moreover, when disturbed or removed from their habitat, Nephtyids display notable swimming abilities (Rouse & Pleijil, 2001). Typically characterized by an elongated body comprised of numerous rectangular segments, these organisms are commonly observed to possess a white coloration. Their slender form features a quadrangular cross-section, with a pair of antennae and short palps adorning the body (Blake & Hilbig, 1990).

Additionally, the pharynx of Nephtyids is eversible and adorned with numerous papillae, while also being equipped with lateral jaws for feeding purposes. The parapodia of these organisms exhibit a biramous structure, showcasing well-developed pre- and postsetal lobes (Fauchald, 1977). Setae, the bristle-like structures found on parapodia, are typically simple in structure, with interramal respiratory cirri commonly present across species. Positioned against the rectangular side of the body, the parapodia house gills within the gap between the notopodium and neuropodium (Ravara & Carvalho, 2017). Furthermore, the pre-acicular row of setae comprises barred or laddered capillaries, while the post-acicular row primarily consists of long capillaries, often minutely denticulate on one side, although forked setae may also be present (Fauvel, 1923). Figure 2.4 provides an overview of a Nephtyidae family member.

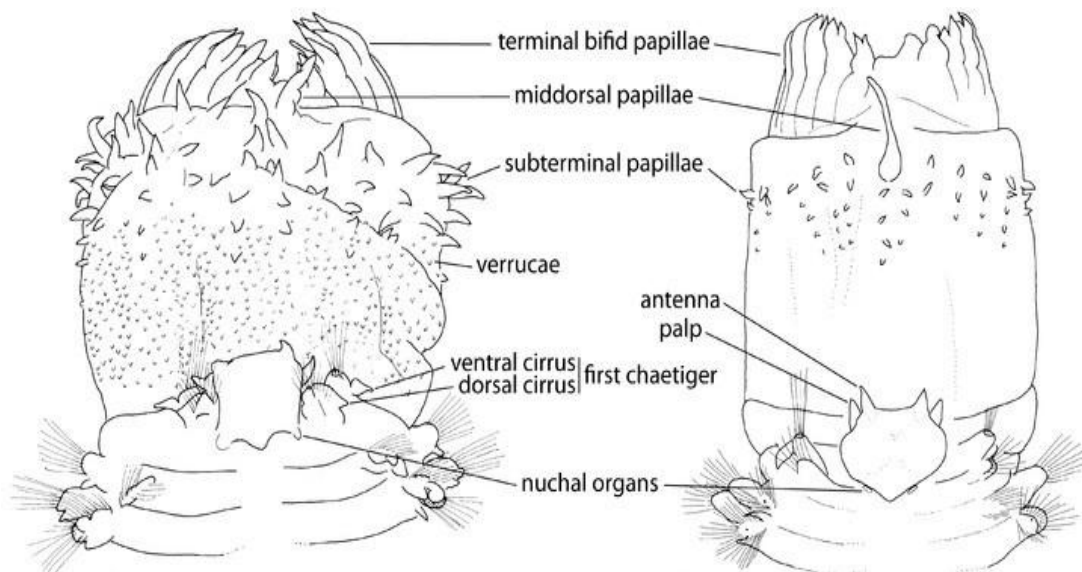


Figure 2.4: Example of Nephtyidae from Handbook of Zoology Online (Ravara & Carvalho, 2017)

2.2.4 Family Onuphidae Kinberg, 1865

The Onuphidae family are tubiculous scavengers that construct a diverse array of tubes and consume a wide range of food items (Fauchald, 1997). Tube composition often correlates with available materials, resulting in translucent tubes, fragile debris tubes, tough muddy tubes, or decorated tubes with shell fragments or leaves, as observed in certain *Diopatra* species (Fauchald & Rouse, 1997).

Common morphological traits observed in this family consist of a prostomium bearing two globular palps, along with two short frontal antennae and five longer occipital antennae mounted on ringed ceratophores (Fauchald, 1997). Well-developed jaws, comprising multiple pairs of lateral maxillae and one pair of lower mandibles, are characteristic, with tentacular cirri present in some species (Fauchald, 1968). Anterior parapodia commonly exhibit a forward orientation and are characterized by tapered ventral cirri, whereas posterior parapodia are comparatively smaller and exhibit cushion-like ventral cirri. Tapered dorsal cirri are consistently observed on all setigerous segments, with notopodia typically manifesting as the base of the branchiae and dorsal cirri, often supported by internal acicula (Çinar *et al.*, 2014). Branchiae may be present, exhibiting simple, pectinately branched, or spirally arranged filaments. Setae types include compound and pseudo-compound hooks, spinigers, pectinate setae, limbate setae, and subacicular hooks (Çinar *et al.*, 2014).

Many species within the Onuphidae family are tubiculous, often characterized by large individuals. Pseudocompound hooks, primarily located on the first few feet, are among the most important setae (Lavesque *et al.*, 2017). Figure 2.5 provides an overview of *Diopatra microcensis* from the Onuphidae family.

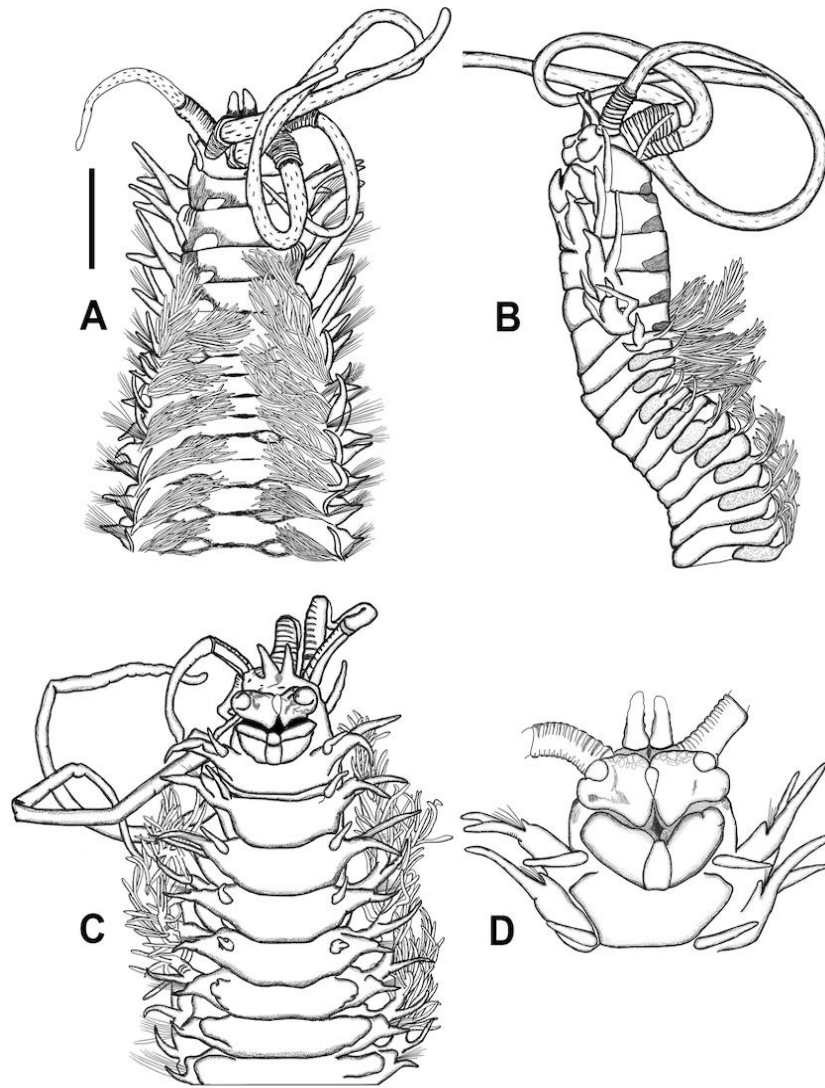


Figure 2.5: Example of Onuphidae (*Diopatra microcensis* from Çinar *et al.*, 2014).

2.2.5 Family Sabellidae Malmgren, 1867

The sabellids, typically tubicolous, are entirely dependent on suspended food particles for nutrition (Rouse & Fauchald, 1997). Constructing sturdy tubes lined with mucoprotein and covered with mud, sand, or debris, they often anchor the base of their tubes in crevices or bore holes in soft corals (Capa *et al.*, 2011). Functioning as suspension feeders, sabellids utilize a pair of branchial lobes adorned with bipinnate radioles to collect suspended particles (Rouse & Fauchald, 1997). These particles, ensnared in mucus, are transported down an inner groove of each radiole to a ciliated sorting groove, where they are directed via ciliated palps to the mouth, guarded by dorsal and ventro-lateral lips. During feeding, a sabellid extends its head out of the tube mouth, distending its trumpet-shaped branchial crown (Rouse & Fauchald, 1997).

Key taxonomic characteristics of sabellids include structures associated with the branchial crown, collar, and setae (Capa *et al.*, 2011). The branchial crown comprises two lobes, each adorned with a semi-circle of bipinnate radioles, often surrounded by an elevated fold or half collar at the base (Tovar-Hernández *et al.*, 2007). The prostomium commonly undergoes reduction and fusion with the peristomium, resulting in the formation of a substantial tentacular crown. The body adopts a cylindrical shape, characterized by a thorax with a limited number of chaetigers and an abdomen exhibiting varying numbers. Notopodia typically carry capillary and/or spatulate setae, while neuropodia showcase uncini, either crested or with teeth arranged in multiple rows. Thoracic uncini may exhibit either a two-row or single-row arrangement, with setal positions reversed on the abdomen. Most species are equipped with tubes constructed from diverse materials, though never composed of calcareous substances (Tovar-Hernández *et al.*, 2007). Figure 2.6 illustrates an overview of *Chone arenicola* from the Sabellidae family.

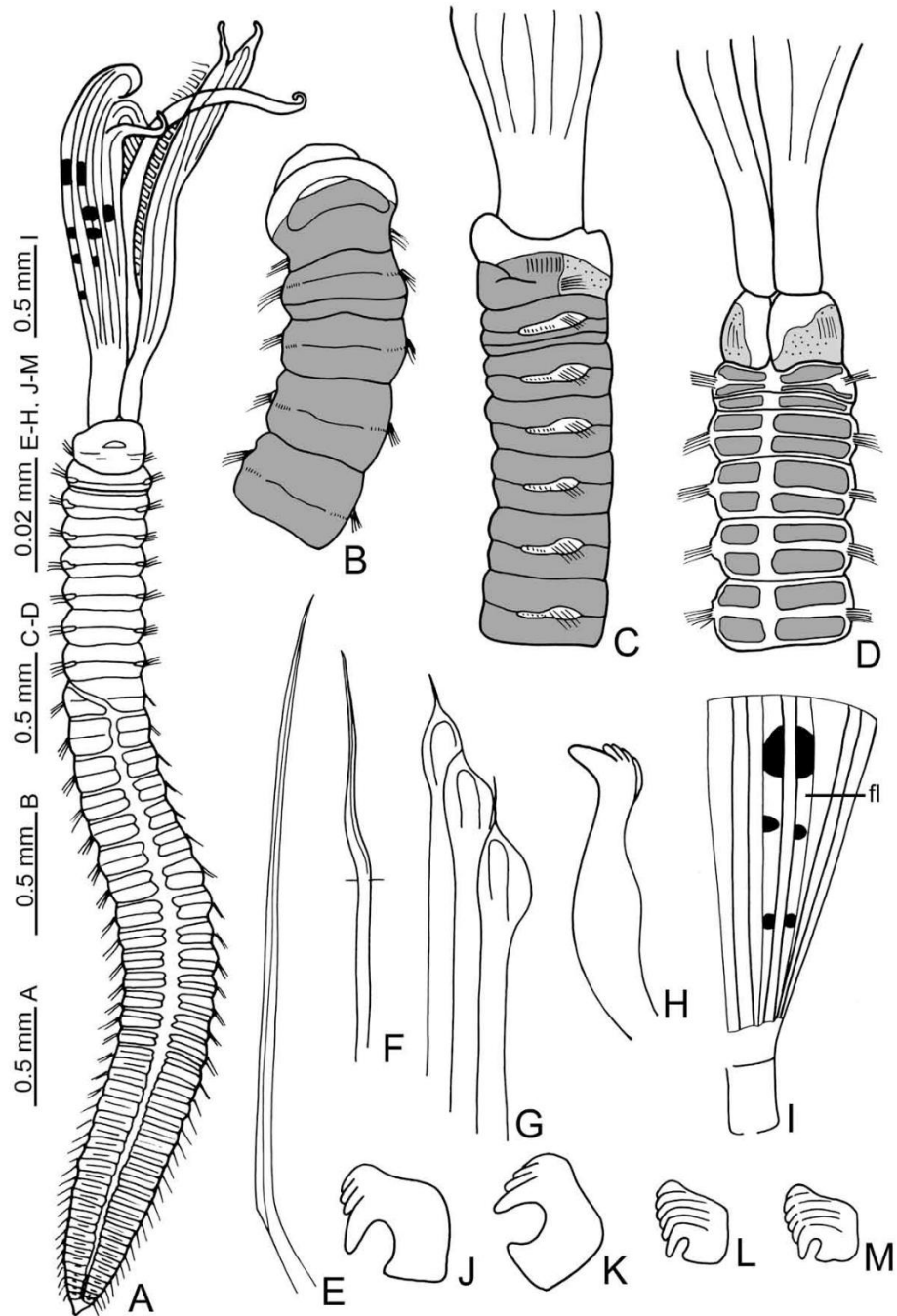


Figure 2.6: Example of Sabellidae (*Chone arenicola* from Tovar-Hernández *et al.*, 2007).

2.2.6 Family Spionidae Grube, 1850

The spionids are recognized as typical deposit feeders, with all members inhabiting burrows lined with a delicate mucoid secretion (Blake, 1996). Spionid utilized their elongated, pointed snouts to engage in sediment excavation. Characteristically, spionids possess a pair of long peristomial tentacles or "palps," which they extend over the substratum to gather food particles (Nygren *et al.*, 2018). Their bodies are vermiform, rather flattened, and comprised of numerous segments, lacking distinct regions except for variations in shape and occasional lateral projections, but devoid of true antennae (Nygren *et al.*, 2018).

Each spionid typically features a pair of long, grooved peristomial palps and an unarmed proboscis (Blake, 1996). Dorsal branchiae are often strap-like or united to the notopodial lamellae, while neuropodia manifest as lateral ridges or lamellae along the elongate body. The prostomium varies, being either blunt with frontal horns or pointed, sometimes featuring an occipital papilla, with other appendages typically absent (Radashevsky, 2023). Parapodia are biramous, with cirriform or foliose lobes, and all setae are simple, including capillaries and hooded or non-hooded hooks, which may be bi- or multidentate (Nygren *et al.*, 2018). The pygidium exhibits anal cirri or a glandular ventral cushion (Radashevsky, 2023). Figure 2.7 provides an overview of *Aonides orensanzi* from the Spionidae family.

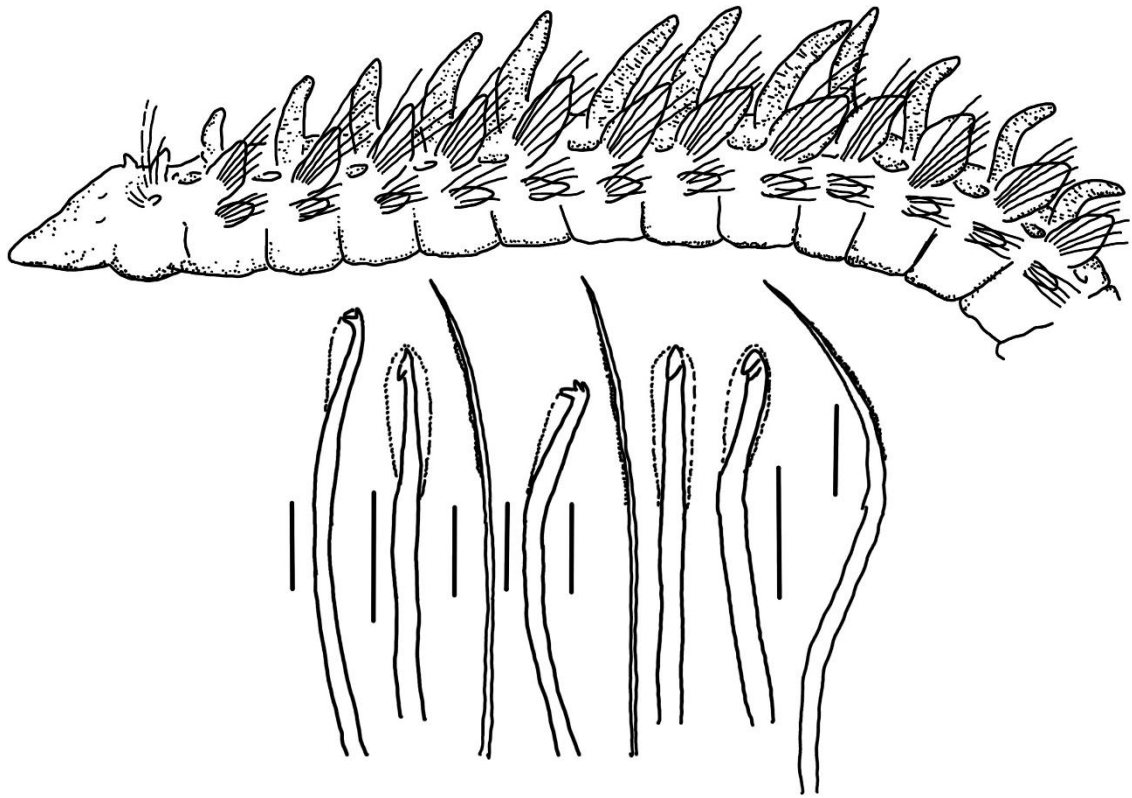


Figure 2.7: Example of Spionidae (*Aonides orensanzi* from Radashevsky, 2015)

2.3 Polychaete Distribution

Polychaetes, a diverse group of marine worms, play critical roles in marine ecosystems owing to their abundance, diversity, and ecological functions. Understanding their distribution patterns is pivotal for evaluating biodiversity, ecosystem health, and addressing environmental changes. Polychaetes demonstrate a global distribution, inhabiting various marine habitats from intertidal zones to deep-sea trenches. Studies by Fauchald (1997) and Rouse & Pleijel (2001) contributed significantly to our understanding of polychaete distribution patterns. The research provided valuable insights into the global distribution of polychaetes, shedding light on their remarkable adaptability and evolutionary success in different marine environments. Building upon these foundational studies, Briggs (1974) and colleagues investigated latitudinal gradients in polychaete diversity. Additionally, the findings revealed higher species richness in tropical and subtropical regions compared to polar areas, with temperature, productivity, and habitat complexity identified as key influencing factors.

The South China Sea, with its diverse coastal habitats, has been a focal point for polychaete research. Numerous studies have significantly enhanced our comprehension of polychaete diversity, taxonomy, and distribution within this region. Al-Hakim and Glasby (2004) investigated polychaete diversity in the Natuna Islands, South China Sea, identifying 52 Linnaean-named species. Of these, six species (12%) were endemic to the South China Sea, while 17 species (33%) were found in both the South China Sea and the neighboring Indo-Malayan peninsula. Additionally, 41 species (79%) were endemic to the Indo-West Pacific region.

Glasby *et al.* (2016) conducted a comprehensive polychaete diversity study in the South China Sea, recording a total of 1257 species (across 73 families) of Annelida. Among these, 289 species were originally described from this region. Nereididae (134 species), Syllidae (100), Polynoidae (76), Serpulidae (72), Spionidae (60) and Eunicidae (59) families exhibited the highest levels of diversity in the South China Sea.

Rosli *et al.* (2016) focused on the continental shelf of the southern South China Sea, identifying 368 polychaete species distributed across 217 genera and 47 families. The Spionidae family was found to be the most prevalent among the collected specimens. Zhang and Hutchings (2018) further contributed to the field by discovering three new species of *Terebellides*, namely *T. guangdongensis* **n. sp.**, *T. yangi* **n. sp.**, and *T. ectopium* **n. sp.**, along the shores of northern South China Sea. These studies collectively underscore the ongoing exploration of polychaete diversity and taxonomy in Southeast Asia, significantly advancing our understanding of these crucial marine organisms.

Furthermore, studies by Hutchings (1998) and Lavesque & Jumars (1999) elucidated regional differences in polychaete diversity and the distinct assemblages of polychaetes in biogeographic regions such as the Indo-Pacific, Atlantic, and Arctic. The study offered a valuable insight into the factors driving spatial variations in species abundance and community structure. In addition to horizontal distribution patterns, Levin & Bridges (1995) and Blake (2000) explored the vertical zonation of polychaetes within marine habitats. Their studies documented depth-related changes in polychaete community composition, highlighting factors such as light availability, temperature, and substrate type as key drivers of vertical distribution patterns.

Distribution of Polychaete along Intertidal area

Polychaeta, a class of Annelida can be generally found on beaches, intertidal areas, soil sediment, marine biome, and reefs. Polychaete make up a high percentage of the common benthic fauna and are a dominant component of macrobenthos in tropical intertidal habitats such as the Malacca Strait, South China Sea, Andaman Sea, Coast of Thailand (Froján *et al.*, 2006) and Songkhla lake (Pattaratumrong & Wongkhamhaeng, 2019).

The diverse habitat preferences exhibited by polychaetes, ranging from soft sediments to hydrothermal vents and cold seeps, were highlighted by numerous researchers (Levin, 2001; Glover *et al.*, 2008). Kostina and Tsurpalo (2018) conducted studies in the intertidal zone of Kunashir Island which shows tropical-boreal or low-boreal and tropical-subtropical species of polychaetes are distributed worldwide from the tropic waters of World Ocean (Indo-Pacific, tropical eastern Pacific, and tropical Atlantic). Whereas in Southern Nigeria, Onwuteaka (2018) examined the habitat preferred by polychaetes in the intertidal and subtidal zone. Musale and Desai (2011) studied the diversity and distribution of soft-bottom polychaetes along the southeast and west coasts of India. In the same year, Lutaenko (2011) studied the coastal areas of the South China Sea, focusing on collecting and compiling information on overall species diversity in coastal areas and islands' ecosystems of Vietnam.

According to Snelgrove (1998), sandy beaches in Sarawak are directly exposed to wave movement whereas mudflats are sheltered and receive fewer action waves. Therefore, the habitat requires the fauna to adapt to the wave action and instability of the area. The middle and lower zones are inhabited by fauna such as burrowing polychaetes, oligochaetes, sipunculans, bivalves, and echinoderms (Long, 2010).

2.4 Polychaete Study in Malaysia

The documentation of polychaetes in Malaysia remains notably deficient despite its rich representation in the marine environment (Glasby *et al.*, 2007). One of the earliest polychaete studies conducted in Malaysia in the early 1990s is by Rullier (1976) which focuses on the findings of polychaete species in Malaysian waters and the identification of new species found. A few decades later, wider polychaete-related research has been conducted in various locations in Malaysia which resulted in many newly-found species around Malaysia (Idris & Arshad, 2013; Idris *et al.*, 2014; Ibrahim *et al.*, 2019). A comprehensive inventory of polychaetes in Malaysia, conducted by Idris and Arshad (2013), provides a chronological account of species records spanning from 1866 to 2013. Since 1866 through 2019, a total of 57 polychaete species have been documented in a revised checklist of polychaete species from Malaysia by Shah *et al.* (2023). This checklist has been updated with integration of species listed in the previous compilation by Idris and Arshad (2013), several new species and records documented by Idris *et al.* (2014), Nishi *et al.* (2015), and Ibrahim *et al.* (2019), and the exception of a few species deemed uncertain due to lacking taxonomic description and validation. However, it is noteworthy that certain records were overlooked, and many species were erroneously classified as cosmopolitan, reflecting the prevailing paradigm until the late 1980s, as noted by Salazar-Vallejo (2020) and Salazar-Vallejo and González-Vallejo (2020).

A total of 121 polychaete species belonging to 37 families are listed in the waters surrounding Straits of Malacca, east of Sumatra, west of Borneo, east of Peninsula Malaysia, Singapore, Natuna Island (Glasby *et al.*, 2016). A compilation of various previous publication of polychaete distributions across South China Sea by Glasby *et al.* (2016) concluded the highest diversity of polychaete family recorded in the South China Sea was

from Nereididae, followed by Syllidae, Polynoidae, Serpulidae, Spionidae and Eunicidae. Nineteen polychaete species from the South China Sea are directly utilized by humans, with 12 of these species commonly harvested by commercial fishermen as baitworms. These include *Antillesoma antillarum* (Grube, 1858), the *Namalycastis abiuma* species group (Glasby *et al.*, 2007), *N. rhodochorde* (Glasby *et al.*, 2007), *Marphysa moribidii* (Idris *et al.*, 2014), *Paraleonnates uschakovi* (Khlebovich & Wu, 1962), *Perinereis aibuhitensis* (Grube, 1878), *Perinereis brevicirris* (Grube, 1878), *Perinereis mictodonta* (Marenzeller, 1879), *Perinereis nuntia* (Savigny in Lamarck, 1818), *Perinereis quatrefagesi* (Grube, 1878); *Halla okudai* (Imajima, 1967) and *Diopatra clapedii* (Grube, 1878).

Buntal Bay, one of the intertidal flats in Sarawak, serves as an important fishery area for important economic species such as razor clam *Solen* spp. (Siti, 2011). An ecological study at Buntal Bay, Sarawak to determine the abundance of macrobenthos and its relation with the environmental factors (Zakirah *et al.*, 2019) has resulted in the findings of various macrobenthos including a few polychaetes; *Barantolla* sp., *Glycera* sp. 3, *Nephtys sphaerocirrata*, *Nephtys* sp. 1, *Nephtys* sp. 2, *Notomastus lineatus*, *Prionospio* sp. 1, *Spiophanes* sp. 1 and *Magelona* sp. 1.

2.5 Molecular Approaches on Polychaete

Despite their ecological significance, polychaetes have received limited attention in DNA studies, primarily due to the challenges of morphology-based species identification (Coull, 1999). In many benthic investigations, polychaetes are often identified only at the family or genus level, as evident in the Barcode of Life Data System (BOLD) database, where only 26,453 out of 38,308 records include species names, representing 3,788 distinct species (accessed June 9, 2024). Taxonomists have acknowledged the difficulties of identifying species morphologically, compounded by factors like the scarcity of taxonomic experts, incomplete identification keys, and the collection of degraded specimens (Rouse & Pleijel, 2001; Knowlton, 1993). These issues are further complicated by cryptic species and complex life stages (Knowlton, 1993; Jarman & Elliott, 2000; Bickford *et al.*, 2007; Nygren, 2014), leading to an increased reliance on expert knowledge for species identification.

Conventional taxonomy often overlooks or misidentifies polychaetes due to factors like colour polymorphisms (Nygren & Pleijel, 2011; Nygren *et al.*, 2011) and the declining number of taxonomists (Hopkins & Freckleton, 2002; Gotelli, 2004; Raven, 2004). Given the limitations of morphological identification, molecular techniques such as PCR and DNA sequencing are being recognized as essential for species delimitation and understanding biodiversity (Zhou *et al.*, 2010; Westheide & Schmidt, 2003; Wörheide *et al.*, 2005; Witt *et al.*, 2006). DNA taxonomy has proven valuable for identifying known species, discovering new ones, and recognizing cryptic species. Phylogenetic reconstruction using gene trees can aid in species delimitation, though mitochondrial genes alone may not fully resolve species relationships. Comparing mitochondrial and nuclear gene trees is a recommended approach to constructing accurate species trees (Achurra & Erseus, 2013; Moore, 1995).

2.5.1 Mitochondrial COI gene

The mitochondrial cytochrome c oxidase subunit I gene (COI) is widely recognized as a valuable marker for species identification in animals due to its ease of amplification across most taxonomic groups and its rapid evolution, which allows differentiation between closely related species. Despite its high mutation rate, the amino acid sequence of COI is highly conserved, making it a robust tool for barcoding (Hebert *et al.*, 2003). Hebert *et al.* (2003) proposed that COI could serve as the foundation for a global bio-identification system for animal life, providing a reliable, cost-effective, and accessible solution for species identification. Once fully implemented, such a system would generate new insights into biodiversity, the diversification of life, and the rules of molecular evolution.

In comparison to other mitochondrial DNA (mtDNA) regions, COI exhibits a higher rate of molecular evolution, which enhances its capacity to discriminate closely related species (Vrijenhoek, 1994). It may also offer superior phylogenetic resolution compared to other regions such as cytochrome b (Cyt b), due to the greater conservation of its amino acid sequences, with most nucleotide substitutions occurring at the third base of each codon (Simmons & Weller, 2001). The robustness of universal primers developed by Folmer *et al.* (1994) enables the amplification of the same COI gene segment across a wide range of species, facilitating comparative studies. Consequently, COI has been employed extensively for species identification (Hebert *et al.*, 2004; Hajibabaei *et al.*, 2005; Brasier *et al.*, 2017; Castro Álvarez *et al.*, 2023), early detection of agricultural pests (Floyd *et al.*, 2010; Pieterse *et al.*, 2010), and monitoring invasive species (Smith *et al.*, 2010).

COI has proven particularly useful in the study of polychaete species, where several studies have confirmed its efficacy in distinguishing species (Rice *et al.*, 2008; Olson *et al.*,

2009; Pleijel *et al.*, 2009; Barroso *et al.*, 2010; Nygren & Pleijel, 2011). It has been widely used in polychaete research for species identification, phylogenetic reconstruction, population genetics, biogeography, and conservation genetics.

For example, Amiri *et al.* (2020) established a COI barcode library for Nereididae polychaetes in Iran. Their research revealed that many previously considered single Nereid populations in the Persian Gulf were, in fact, composed of two or more distinct lineages. Sun *et al.* (2018) utilized COI barcoding to investigate the global phylogenetic and biogeographic relationships of the polychaete genus *Hydroides*. This molecular marker enabled rapid and effective species identification, contributing to the discovery of high genetic diversity within *Hydroides* and suggesting the presence of cryptic species. These studies demonstrate the power of COI barcoding in resolving taxonomic ambiguities and uncovering hidden diversity within polychaete populations. Additionally, the versatility of COI has allowed researchers to further unravel the complexities of polychaete diversity and biogeography (Chen *et al.*, 2021).

However, despite its many advantages, COI has limitations when used for species identification. In some cases, intraspecific variation may exceed interspecific differences, complicating species designations (Meyer & Paulay, 2005; Vences *et al.*, 2005; Hill *et al.*, 2012). Other potential issues include the retention of ancestral polymorphism, sex-biased gene flow, hybridization, introgression, and the presence of nuclear copies of mitochondrial genes (Moritz & Cicero, 2004; Hebert *et al.*, 2004; Rubinoff *et al.*, 2006). In other cases, low levels of intraspecific variation have rendered COI ineffective for resolving phylogenetic relationships among closely related species (Meier *et al.*, 2006; Huang *et al.*, 2008).

Despite these challenges, researchers have successfully amplified COI sequences from various polychaete taxa using universal primers for invertebrates, although the success rate can vary. For example, PCR amplifications from Zhou *et al.* (2010) used universal COI primers (HCO2198 and LCO1490, developed by Folmer *et al.*, 1994) alongside 16S rDNA primers, and although deep phylogenies above the genus level were unresolved, most congeneric relationships were well-supported. However, relationships among *Nereis*, *Perinereis* and *Platynereis* remained unresolved. Technical challenges continue to affect the amplification of COI sequences in some polychaete taxa, with Carr *et al.* (2011) reporting an 81% success rate when using three COI primer sets to amplify barcode regions from 1,876 of 2,324 specimens. Modifications to primer sets, such as the polyLCO/polyHCO set, have achieved roughly 70% success (Carr *et al.*, 2011), while Rice *et al.* (2008) encountered amplification difficulties with Folmer's universal primers under various conditions. To address these issues, researchers have developed specific COI primers for *Polydora cornuta*, though only one pair has been formally published (Takata *et al.*, 2011).

2.5.2 Mitochondrial 16S rRNA gene

The 16S gene marker has been widely applied in molecular analyses of polychaete species, with numerous studies confirming its reliability (Liline, *et al.*, 2016). For example, Aguado *et al.* (2007), Tosuji & Sato (2008), Zhou *et al.* (2010), and others demonstrated the successful amplification and sequencing of 16S rRNA across various polychaete species, enhancing the understanding of phylogenetic relationships and species delimitation in this group. Guggolz *et al.* (2020), for instance, identified 21 lineages of *Prionospio* and *Aurospio* from the deep Atlantic and Pacific Oceans using the 16S rRNA gene. These lineages were consistently resolved using 16S, showing its robustness for species delineation, particularly in taxa that inhabit diverse or deep-sea environments. Zhou *et al.* (2010) also found that for

species like *Nereis vexillosa*, where the universal COI primer failed to yield amplification, the 16S rDNA marker successfully confirmed species identification. This finding highlights the utility of 16S as a complementary molecular marker, particularly in cases where COI might face limitations. According to Hajibabaei *et al.* (2007), the 16S rDNA is especially effective for studying closely related species or genera, despite its slower evolutionary rate compared to COI. Its ease of amplification and sequencing makes it highly applicable, although aligning sequences from divergent taxa can be more challenging due to the presence of indels and gaps.

Studies have utilized the 16S rRNA gene to reconstruct the deep phylogeny of polychaetes, resolving species relationships within genera, assessing genetic diversity within populations, and investigating population structure. For instance, Struck *et al.* (2006) uses 16S rRNA and nuclear markers to address deep phylogenetic relationships within annelids. It provides one of the earlier molecular frameworks for resolving polychaete phylogeny. Santos *et al.* (2006) focused on phylogenetic relationships within the Nereididae family using mitochondrial 16S rRNA genes, contributing insights into evolutionary relationships in polychaetes. Beyond these applications, the 16S rRNA gene has also been used to examine co-evolutionary patterns between polychaetes and their associated symbionts. Hourdez *et al.* (2021) investigated the symbiosis in *Capitella* species, using both 16S rRNA and other genetic markers to understand host-symbiont relationships, which include species differentiation in the genus. In conclusion, the 16S rRNA gene offers a versatile approach for studying polychaete DNA, providing valuable insights into their evolutionary history, population dynamics, and interactions with symbiotic organisms. While it may not be as widely used for direct species identification as the COI gene, its utility in understanding the broader context of polychaete biology is undeniable.

2.5.3 28S Ribosomal DNA

The 28S rDNA gene is located between the ITS2 gene and ETS, and almost all eukaryotes have hundreds of copies of the ITS gene, which show more divergence than their flanking regions and are easily amplified (Cheng *et al.*, 2006). The region is frequently used in specific identification to differentiate between related species or to infer the phylogenetic relationships from populations to families (Hwang & Kim, 1999) and even higher taxonomic levels (Coleman & Vacquier, 2002). Hwang and Kim (1999) mentioned that the 18S rDNA gene can differentiate organisms from Kingdom to Genus, but the 28S rDNA gene can differentiate organisms from Class to Species level. Previous studies have recorded successful results in the utilization of the 28S gene for molecular analyses of polychaete species in supporting morphological identification (Patti *et al.*, 2003; McHugh, 2005; Jimi *et al.*, 2021; Kin *et al.*, 2022; Kupriyanova *et al.*, 2023; Lim *et al.*, 2023). For instance, Rousset *et al.* (2003) examined the phylogenetic position of Alvinellidae, a group of polychaetes known only from hydrothermal vents and initially categorized as a subfamily of Ampharetidae (Desbruyeres & Laubier, 1980), using sequences of 28S rRNA (D1 domain) and 52 morphological characters.

The 28S rRNA gene, while less frequently used for polychaete DNA identification than COI or 16S rRNA due to its lower sequence variability, has proven valuable when combined with other genetic markers. Several studies have demonstrated its utility in investigating population genetics, phylogeography, and conservation status. Ruta *et al.* (2007) assessed phylogenetic relationships within the Hesionidae family using a combination of morphological data and nucleotide data from 18S rDNA, 28S rDNA, 16S rDNA, and COI. When combined with other markers, 28S rRNA can aid in resolving phylogenetic relationships that are challenging to elucidate using COI or 16S rRNA alone.

Weigert and Bleidorn (2016) provided a comprehensive review of annelid phylogeny, emphasizing the importance of combining various molecular markers, including 28S rRNA, with morphology and other genetic markers like COI and 16S. This study clarified the placement of previously unresolved taxa, concluding that combining 28S rRNA with faster-evolving genes like COI or 16S rRNA enhances the resolution of deep phylogenetic relationships within annelids. Its slower evolutionary rate can be advantageous for studying deep-level phylogenetic relationships within polychaete taxa.

Rouse *et al.* (2024) recently investigated the evolutionary transitions of brooding modes within the Spirorbinae subfamily of Serpulidae. Employing a combined dataset of molecular (28S and 18S rDNA) and morphological (83 characters) data for 36 taxa, they reconstructed the phylogeny of Spirorbinae. Their analysis strongly supported the monophyly of Spirorbinae and five of its tribes (Circeini, Januini, Romanchellini, Paralaeospirini, and Spirorbini), while challenging the monophyly of Pileolariini. These studies collectively demonstrate the potential of 28S rRNA to make substantial contributions to polychaete research. By providing insights into population dynamics, evolutionary history, and conservation needs, 28S rRNA can effectively complement the information derived from other genetic markers. When used in conjunction with COI or 16S rRNA, 28S rRNA can enhance the accuracy and comprehensiveness of polychaete DNA identification and analysis.

2.6 Economic Value

The benefit of polychaete for the aquaculture sector has received much attention from scientists over the past decades. Numerous researches on polychaete has been focused on identifying efficient feed that enhances its optimal growth and survival. Shi *et al.* (2014)

stated that polychaetes are a primary food source for other higher-order organisms in the food web and polychaetes are useful organisms that are used for research studies. Polychaete are an essential source of food for demersal fish, polychaetes play a significant role in the marine food chain, particularly for bottom fish and some mammals (Parulekar *et al.*, 1982; Herman *et al.*, 2000). The fatty acids in polychaetes are being used as food for crustaceans and fish as it is essential in the development of the gonads.

Polychaetes utilize all available food sources within the sediment and serve as a crucial link in energy transfer. Benthic fauna, in general, play a vital role in the trophic system (Crisp *et al.*, 1971; Sivaleela & Venkataraman., 2012). Polychaetes are integral to the food web and play a significant role in the burial of organic matter, thereby regulating the flux of organic carbon from the global oceans to the atmosphere (Diaz & Breitburg, 2009). Dean (2008) claims polychaetes are used as indicators to indicate the diversity of benthic species, organic enrichment, level of heavy metal pollution, and organic contaminant.

Commercial fishermen utilize polychaetes as bait in commercial fishing (Cabral *et al.*, 2019). Furthermore, some polychaete species can be economically exploited, either directly (as baitworm) (Olive, 1994) or indirectly (as aquaculture feed for broodstock) (Olive, 1999). In Portugal's Canal de Mira Estuary, for instance, the *Diopatra neopolitana* fishery is valued at €325,000 per year (Cunha *et al.*, 2005), while in Maine, USA, the collection of bloodworms (*Glycera alba*), which has produced 800 jobs annually since 1940, has an average catch value of USD 6 million (Sypitkowski *et al.*, 2009), generates 800 jobs annually.

CHAPTER 3

GENERAL METHODOLOGY

3.1 Sampling Sites

Buntal beach is located in the Southern part of Sarawak, East Malaysia and Borneo waters. The sampling sites were located between coordinate 1°41'53' N, 110°22'24" E and 1°42'53' N, 110°22'43" E. Samples were collected on March and August 2021. Two transects were established along the intertidal zone to capture spatial variability in polychaete distribution and potential impacts of localized factors. Four sampling stations were positioned along each transect, with intervals of 150 meters between stations, allowing for systematic sampling across the intertidal area during low tide (Figure 3.1).

Figure 3.2 provides a schematic representation of the sampling setup at Buntal Beach. The two transects (T1 and T2) were laid parallel to the shore, with each transect starting from the high-tide level (HT) and extending to the low-tide level (LT). Stations along the transects (T1S1–T1S4 for Transect 1 and T2S1–T2S4 for Transect 2) were carefully positioned to ensure that polychaete distribution could be studied across varying tidal heights and sediment conditions.

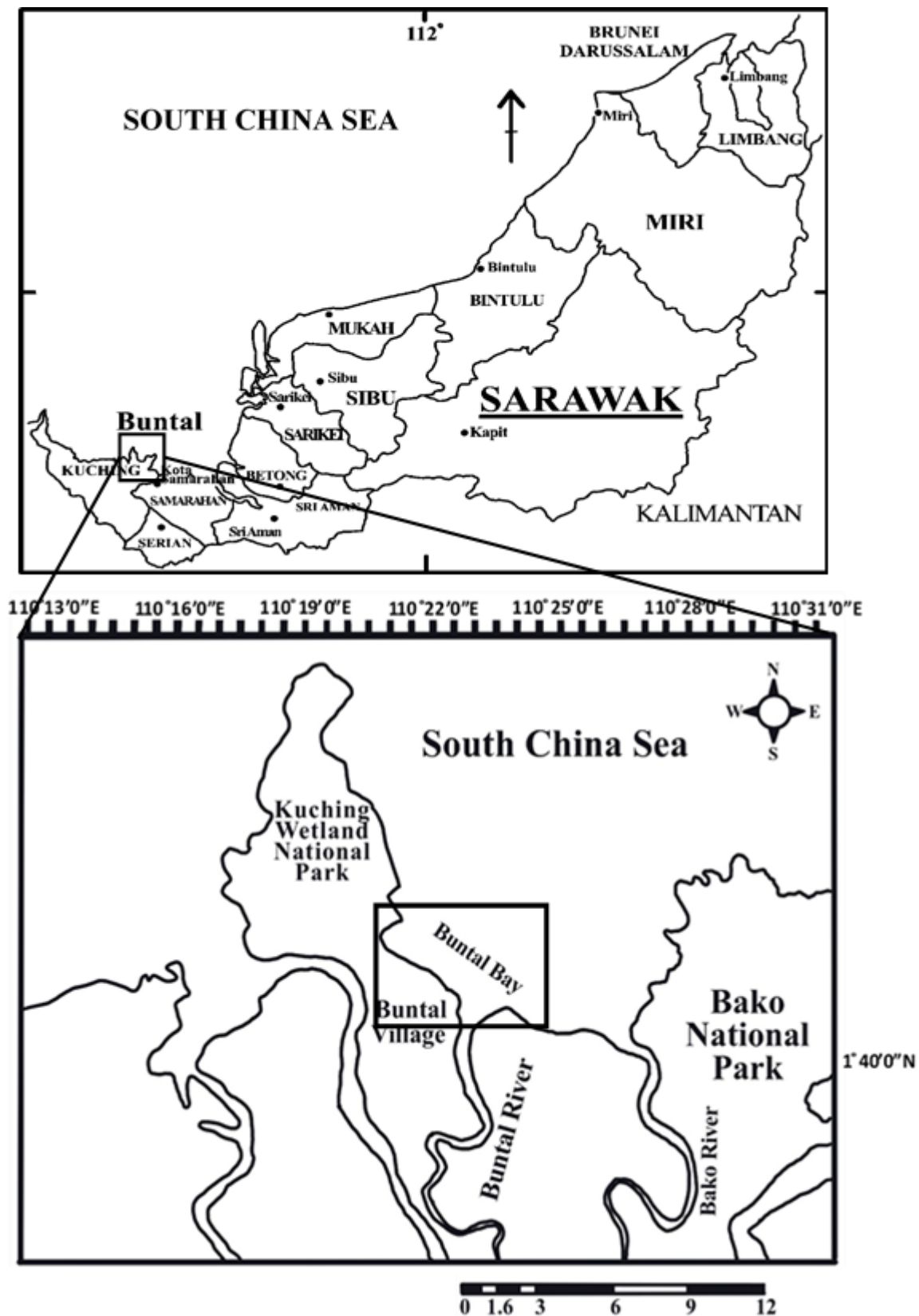


Figure 3.1: Map of Sarawak showing the location of Buntal beach.

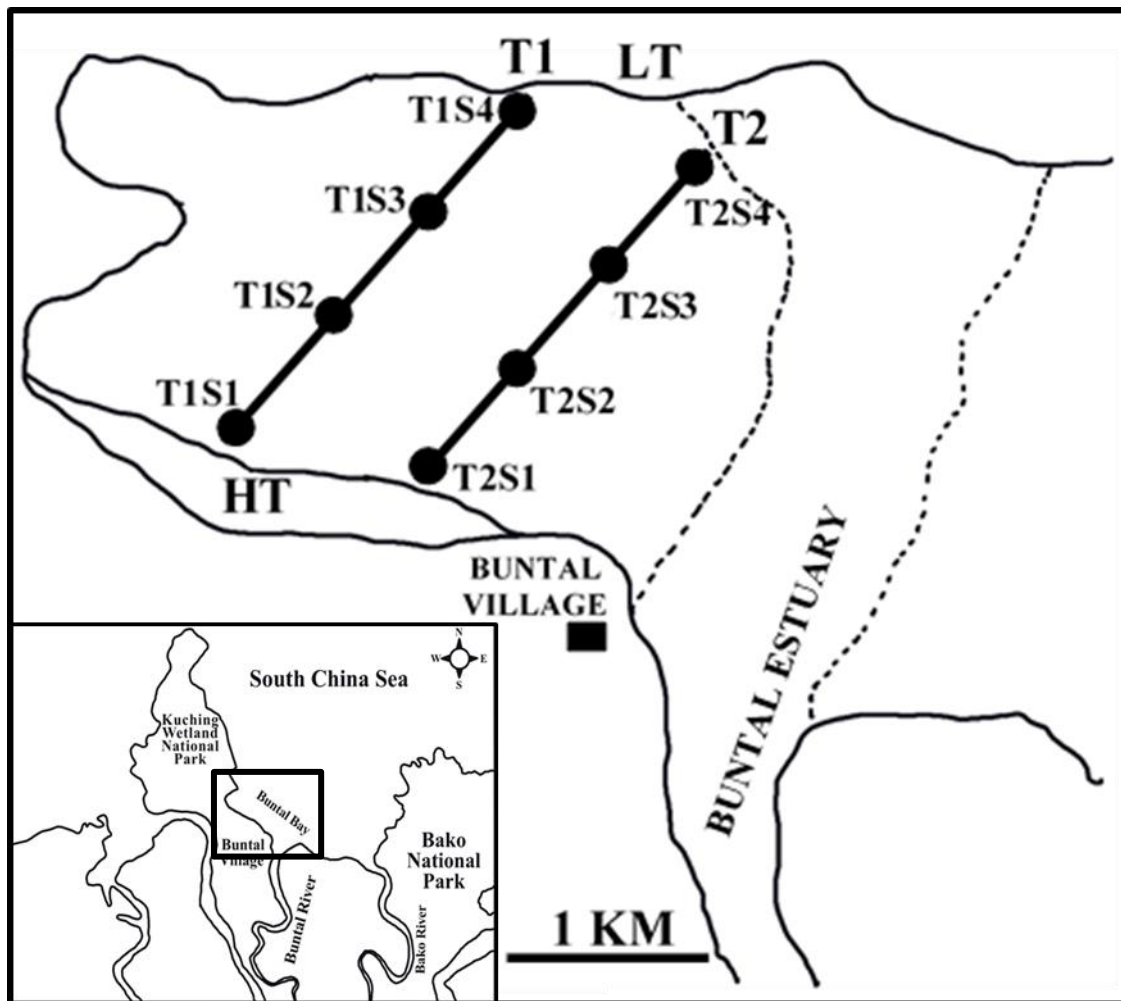


Figure 3.2: Illustrations of line transects performed in the intertidal zone of Buntal Beach. T1=Transect 1, T2=Transect 2, HT=High tide level, LT=Low tide level, T1S1 – T1S4=Station 1 to 4 in Transect 1, T2S1 – T2S4= Station 1 to 4 in Transect 2.

3.2 Sample Collection and Preservation

Two transects were established along the intertidal zone of the beach, spaced 1 km apart. Each transect consisted of four sampling stations, located 150 meters apart from one another (Clarke & Warwick, 1994). Sediment collection was performed during the low tide at each station by using a perspex corer (Crisp *et al.*, 1971).

Methodically, triplicate sediment samples, each measuring 10 cm in depth, were obtained for taxonomy analysis, while an equivalent set of triplicates was obtained for subsequent DNA analysis. Additionally, a singular replicate of sediment, with a depth of 10 cm, was meticulously gathered for sediment analysis (Blott & Pye, 2001). Replication strategy was employed to ensure the reliability and generalizability of the findings. These sampling endeavours were conducted within a predefined quadrat measuring 0.5 m x 0.5 m (Gage & Tyler, 1991). The samples reserved for taxonomy analysis were preserved in a solution of 5% formalin and then transferred to the laboratory. On the other hand, the DNA samples were subjected to fixation in a solution of denatured ethanol, preparing them for further molecular analyses.

3.3 Taxonomic analysis

Sample identification was conducted under a compound microscope, focusing on key morphological features such as parapodia, setae, and head structures. Additional techniques, including dissection of larger specimens and electron microscopy, were employed to capture detailed images of smaller body parts. Photographs were taken, and taxonomic keys and literature were referenced to ensure accurate species identification. Specimens requiring SEM were prepared using a dehydration series of increasing ethanol concentrations (70% to 100%), followed by Critical Point Drying (CPD) using liquid CO₂. The specimens were visualized under electron beams for detailed examination. In parallel, sediment analysis was performed to understand the environmental context. Sediment samples were air-dried, sieved, and the particle size distribution was recorded to investigate habitat characteristics relevant to polychaete distribution.

3.4 Molecular analysis

This study utilized two DNA extraction methods: a modified CTAB protocol and the Qiagen DNeasy Blood and Tissue Kit. The CTAB method involved mincing polychaete samples, followed by chemical treatment with CTAB buffer and Proteinase K to break down cellular components. The Qiagen DNeasy Kit method involved the use of a proprietary set of reagents and apparatus. PCR amplification of three gene regions—COI, 16S, and 28S—was conducted using specific primers. The choice of COI, 16S, and 28S as molecular markers for phylogenetic analysis is well-supported by previous studies in the field of polychaete taxonomy and phylogeny of Eulepethidae (Wolley & Wilson, 2011; Norlinder *et al.* 2012; Zhang *et al.*, 2017). The PCR products were verified through agarose gel electrophoresis, and the successful amplification products were purified using the Norgen Purification Kit before being sent for sequencing.

For phylogenetic analysis, the cleaned and aligned sequences were submitted to BLAST to compare against available Genbank sequences. This comparison helped in identifying closely related sequences and confirming polychaete identification. The study analyzed COI, 16S, and 28S sequences from newly obtained and Genbank data, including outgroup taxa, using multiple sequence alignment tools like MUSCLE and ClustalX. Phylogenetic trees were constructed using Neighbour-Joining (NJ), Maximum Parsimony (MP), Maximum Likelihood (ML), and Bayesian inference methods in software such as MEGA, PAUP, and MrBayes. These analyses aimed to trace evolutionary relationships within the polychaete family Eulepethidae and its relatives, providing insights into their genetic distances and evolutionary history.

CHAPTER 4

IDENTIFICATION OF POLYCHAETE IN BUNTAL BEACH

4.1 Introduction

Conventional morphological identification involves the examination of physical characteristics and structures of organisms. This process is crucial for accurately identifying and classifying species, allowing scientists to distinguish one species from another based on observable traits. Conventionally, species identification has been done by implementing dichotomous identification keys and analysing morphological data (Balakrishnan, 2005). Although keys are built with general users in mind, it has presented a much easier guidelines for experts, and for people who have specialized knowledge of many taxa (Schlick-Steiner *et al.*, 2010). Conventional morphological identification methods are often accessible and does not require advanced technology (Dayrat, 2005). This makes the method to be applicable in a wide range of settings, including fieldwork and locations with limited resources. This method is convenient for in-field sorting or early sorting stage as distinguishable characteristics of certain families can ease the sorting process by referring to the taxonomical drawings of the polychaete (Winston, 1999).

Conventional morphological identification provides baseline data for environmental studies especially focusing in certain geographical area (Bortolus, 2008). By referring to previous taxonomy studies, researchers are able to recognize which species are present in an area and monitor its population changes over time, assessing the impact of disturbances, and making informed conservation decisions. Conventional morphological identification has historical significance, especially in the context of scientific literature. When new species are discovered, conventional morphological identification is often the first step in

characterizing and verifying these species. This method ensures that the new discoveries are properly documented and classified within the existing scientific framework (Wheeler, 2004). Many species have been described and documented based on morphological characteristics, creating a rich scientific heritage and facilitating the comparison of data across different time periods.

4.2 Problem Statement

Previous studies on polychaetes in Malaysia have primarily focused on taxonomy and morphological identification methods, with limited attention given to Sarawak waters, particularly Buntal beach. Existing records show a scarcity of identified species in this region compared to Peninsular Malaysia (Gholizadeh *et al.*, 2012; Idris & Arshad, 2013; Rosli, 2015). For instance, Idris and Arshad (2013) compiled a checklist of annelids in Malaysia, but only one polychaete species was recorded from Sarawak. Similarly, a study by Zakirah *et al.* (2019) identified several polychaete species in Buntal beach. However, the methodology was not exhaustive for establishing a comprehensive species checklist. This lack of comprehensive research may be attributed to insufficient collecting efforts by local taxonomists or scientific expeditions (Paxton & Chou, 2001). Therefore, there is a need for more detailed studies focusing specifically on polychaetes in Sarawak waters.

Identifying polychaetes presents challenges due to various factors. Morphological similarities among species make differentiation difficult, compounded by specimen fragmentation during collection, often resulting in partial remains. Additionally, many polychaete species are small, measuring less than 20 mm in length and under 2 mm in diameter, necessitating the use of a microscope for accurate identification (Rouse & Pleijel, 2001). The intricacies of polychaete identification demand a comprehensive examination of

the front end for most species. In some cases, a complete posterior end may also be necessary, or a meticulous count of segments with specific features like setae, gills, or parapodial lobes may be required (Glasby & Timm, 2019). To further address these challenges, this study employed additional preservation techniques and utilized photography, complemented by the application of Scanning Electron Microscope (SEM). The conventional technique of light microscopes, paired with these advanced techniques of electron microscope were employed to support the identification process and overcome the limitations posed by the small size and potential fragmentation of polychaete specimens.

4.3 Objectives

This chapter aims:

- i. To identify each polychaete collected in the intertidal area of Buntal beach up to their species level
- ii. To create dichotomous key and provide the taxonomical drawings of the polychaetes
- iii. To conduct a comprehensive taxonomic analysis and provide complete morphological description of a novel species in Eulepethidae family

4.4 Materials and Methods

4.4.1 Taxonomy

4.4.1.1 Sample Sorting

The preserved sediment was further processed in the laboratory through a 0.5 mm mesh sieve to separate different particle fractions and remove debris and large organisms. The sieved product was carefully examined under a stereo microscope (Nikon Stereo Microscope SMZ-745) to identify and isolate polychaete specimens. Once polychaete was identified, fine forceps were used to separate them from the sediment matrix and the specimens were placed in a vial with 70% ethanol for further processing and analysis.

4.4.1.2 Sample Identification

Once sorted, polychaete specimens were further examined under a compound microscope for detailed morphological analysis. External features, including body size, shape, colour, and distinctive markings, were examined to identify specimens at the lowest taxonomic level possible. Greater attention was paid to features like parapodia, setae, and head morphology, including antennae, palps, and jaws, which can vary greatly between species and crucial for species identification. The number of body segments was tallied and any variations or modifications noted. For some bigger sample, distinctive body parts such as antennae and parapodia were dissected under stereo microscope by using forceps and blade for observation.

The full body or dissected body parts observed by light microscope were transformed into taxonomical drawing to provide full view of the studied species. Body parts of the smaller samples that were unable to be observed by optical were proceeded to electron microscope for better visualization. Photographs of the specimen were taken from different

angles to capture important features, which can be useful for future reference. Taxonomic literature and monographs related to polychaetes were checked, providing detailed descriptions and illustrations for specific species. Detailed observations, including the collection location and habitat, were recorded. Specimens were catalogued and properly preserved for future reference. The list of catalogue number for the samples of this study were listed in Appendix B, Table 2.

The polychaete was identified with the aid of dichotomous keys from identification books such as “Polychaetes & Allies, The Southern Synthesis” by Beesley *et al.*, (2000), “Polychaetes” by Rouse & Pleijil, (2001) and “Invertebrates” by Brusca & Brusca, (2023) and scientific papers (Zhang *et al.*, 2017; Nygren *et al.*, 2018; Ibrahim *et al.*, 2019; Bonifácio *et al.*, 2020; Radashevsky, 2023), as well as reference collections in museums, research institutions, or online databases specializing in polychaetes (Audouin & Milne-Edwards, 1833; Chamberlin, 1919; Berkeley & Berkeley, 1939; Friedrich, 1939; Wesenburg-Lund, 1949; Dales, 1959; Khlebovich & Wu, 1962; Pettibone, 1963; Sasekumar, 1974; Imajima, 1967; Fauchald, 1977; Shin, 1980; Tan & Chou, 1993; Fauchald & Rouse, 1997; Glasby *et al.*, 2000; Wilson, 2000; Costello, 2001; Levin, 2001; Aungtonya *et al.*, 2002; Bartolomaeus & Purschke, 2002; Wehe & Fiege, 2002; Kumar, 2003; Al-Hakim & Glasby, 2004; Salazar-Vallejo & Londono-Mesa, 2004; Shin *et al.*, 2004; Balakrishnan, 2005; Quijon & Snelgrove, 2005; Speybroeck, 2007; Glover *et al.*, 2008; Musale & Desai, 2011; Mohamad, 2014; Shi *et al.*, 2014; Lavesque *et al.*, 2015; Glasby *et al.*, 2016; Glasby & Hakim, 2017; Gogina *et al.*, 2018; Adkins *et al.*, 2023).

4.4.1.3 SEM Preparation Techniques adapted from Murtey & Ramasamy (2016), with some Modification

The preparation of specimens for scanning electron microscopy (SEM) involves placing them within a high vacuum chamber with a conductive surface to facilitate electron and matter interactions for image formation. In order to obtain a good visualization of the sample, the samples were processed carefully to avoid shrinking or damage to any body parts. Therefore, a pivotal preparation step in SEM analysis were presented in Appendix B: Figure 2.

4.4.1.4 Fixation and Series of Dehydration

The dehydration step was done with immersing the sample in a series of increasing concentrations of ethanol (EtOH). A series of EtOH were implemented on the samples for a certain amount of time to fully remove the remaining formalin or other preservatives without causing the sample to lose its original shape and size or shrink its body parts. Therefore, four concentrations of EtOH were prepared in this study; 70%, 80%, 90% and 100%. EtOH concentration was increased gradually to avoid immediate water removal that could cause specimen shrinkage. The samples were soaked in 70% EtOH for an hour, transferred into 80% EtOH for another hour and later transferred into 90% EtOH for another hour. The samples were then transferred into 100% (absolute) EtOH for an hour and this final step were repeated three times to ensure full water removal from the body. The list of chemicals and series of dehydration steps were tabulated in Appendix B: Table 1.

4.4.1.5 Critical Point Drying (CPD)

Next, it is important to ensure that the sample was dried completely without creating micro-ripping of the surface. As the high surface tension solvent evaporates, the surface structure of the sample could be damaged while it changes from liquid to a gaseous state. The drying of the sample was conducted using Critical Point Drying (CPD) technique where liquid CO₂ serves as the substitution of EtOH to prevent potential stiction upon drying. CPD helps push the liquid CO₂ past its critical point and converting it to supercritical fluid and then converting that fluid to gas. Residual water and EtOH in the sample or its surroundings were completely substituted by liquid CO₂ and pushed out in gaseous form afterward. The samples loaded into the CPD machine undergoes seven to 10 cycles of liquid CO₂ immersion to ensure complete removal of EtOH. Samples were immersed for 10 minutes each cycle before discarding the used CO₂ and refilling new liquid CO₂ into the chamber and starting the next cycle. The liquid CO₂ in the final cycle was not removed after 10 minutes and the temperature were risen to 40°C to convert the remaining liquid to gaseous state. It is crucial to ensure that all the liquid CO₂ converted to gaseous form before releasing it out.

4.4.1.6 Mounting on a plate

The dried samples were mounted on a small piece of metal plate for platinum coating. The process was conducted using 0.5 x 0.5cm² metal plate, larval forceps and carbon tape. The samples were handled carefully after being taken out of the chamber and placed into a petri dish. The samples were examined under a stereo microscope to determine the upper and lower body parts to ensure correct placement onto the plate. After it was determined, the sample was mounted on the metal plate using the carbon tape. Bigger samples were cut into smaller pieces to ease the mounting and coating process. The metal plate was then labelled and proceeded for coating.

4.4.1.7 Sputter Coating with Platinum

Platinum serves as the conductive coating for the visualization of the sample surface. It is important to carefully coat the whole sample to ensure smooth electron flow for complete visualization on all surface area. The mounted samples were fully coated with platinum by a coating machine. Up to three to four samples can be coated at the same time. Platinum serves as a conductive layer for the electron to ease the viewing process during SEM. This coating step was crucial for the electrons to pass through or onto the surface and provide high-magnification 3D imaging of the samples.

4.4.1.8 SEM Visualization

The coated sample were mounted to the SEM chamber. The chamber was depressurized for two minutes, a beam of electrons was shot onto the samples and the samples were viewed on the monitor with desired magnification and definition. The selected parts of the samples were observed for identification purposes.

4.4.2 Sediment Analysis

Sediment analysis plays a pivotal role in polychaete species identification and taxonomy by providing insights into the properties of the substrate that influence the morphological adaptations and body characteristics of these organisms (Blake, 2000). By examining sediment composition, grain size, and organic content, researchers can infer how polychaetes interact with their environment and adapt their body structures for locomotion, feeding, and reproduction (Fauchald, 1977).

The sediment samples obtained from each station were processed for Particle Sediment Analysis. The sediment samples were air-dried to eliminate surplus water. Any aggregates were disintegrated and organic material or debris were eliminated from the sample. 100 g of the prepared sediment samples were used initially. Prior to usage, each sieve was weighted to document their respective weights. The sediment sample was positioned onto the uppermost sieve and subjected to mechanical sieving for 15 minutes. The material retained on each sieve were weighted and recorded. The cumulative weight percentages were calculated for each sieve

4.5 Results and Discussion

4.5.1 Species Composition

Cumulatively, 116 polychaete individuals were obtained from the 8 stations of Buntal beach. T1S1 and T2S1 were in the high tide zones, T1S2, T1S3, T2S2 and T2S3 were in the mid tidal zones and T1S4 and T2S4 were in the low tide zones. Among these stations, 4 stations with >10% percentage of the polychaete count in Buntal beach were identified. Notably, station T2S2 yielded the biggest individual count, constituting 29% of the total sample (n=34). Following closely, station T1S2 contributed 23% of the sample (n=27) while station T2S3 and T1S3 yielded 18% (n=21) and 10% (n=11) of the total sample, respectively. These 4 stations were located in the mid tidal zone of the Buntal beach. The remaining 23 individuals were distributed across other stations, each contributing less than 10% to the total abundance.

In general, the number of polychaetes collected in Buntal beach were higher in stations located in the mid tidal zone (T1S2, T1S3, T2S2 and T2S3) compared to low tidal (T1S4, T2S4) and high tide zones (T1S1 and T2S1). Mid tidal zone, recorded the biggest polychaete individual count, which contributed 80% (n=93) of the total sample of the Buntal samples. Followed by lower intertidal zone, with a total contribution of 12% (n=14) to the Buntal samples. High tidal zone recorded the lowest individual count, making up only 8% (n=9) of the polychaete count.

From the Buntal stations, a total of 116 individuals were collected, revealing 10 species belonging to 6 polychaete families. *Buntalepis sarawakensis* **sp. nov.** was discovered to be the dominant species, with a total of 26 individuals across 5 Buntal stations. *Glycera alba* and *Onuphis eremita* were encountered at 5 and 4 stations, respectively, with 19

individuals each. *Glycera tessellata* appeared in 11 individuals across 6 stations, and *Euchone* sp. 1 was observed in 10 individuals across 3 stations. *Glycera tridactyla* was present in 9 individuals across 6 stations, *Micronephthys sphaerocirrata* in 7 individuals across 3 stations, and *Onuphis* sp. 1 in 7 individuals across 2 stations. *Onuphis* sp. 2 was found in 5 individuals across 3 stations and *Scoelepis* (*Scoelepis*) *squamata* in 3 individuals across 2 stations. Figure 4.1 displays the proportion of the species discovered in Buntal beach.

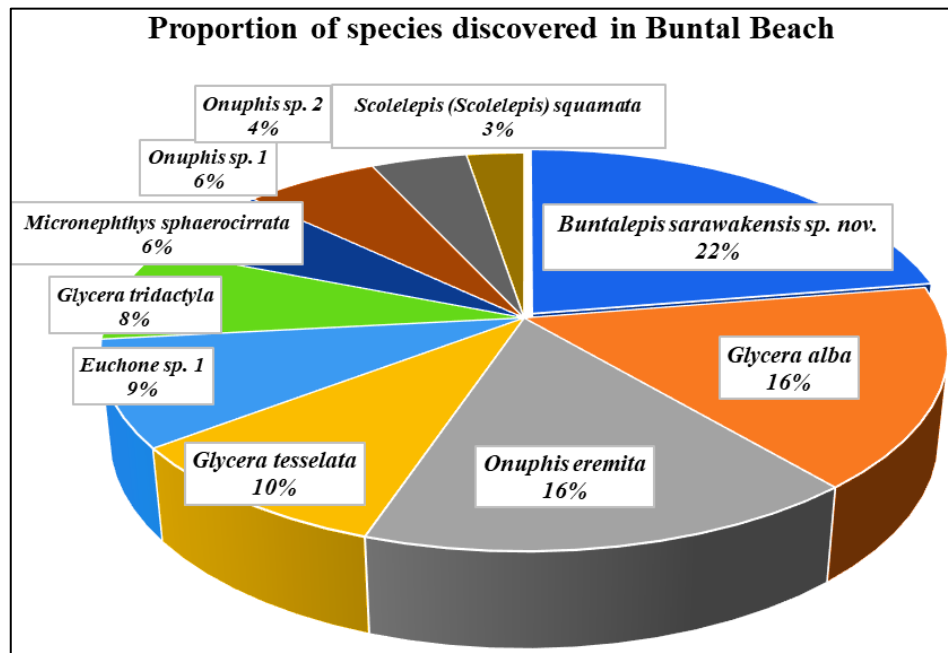


Figure 4.1: Proportion of species discovered in selected stations of Buntal Beach.

T2S2 exhibits the highest polychaete diversity, hosting 9 different species, while T1S2 and T2S3 also demonstrate substantial diversity with eight species each. In contrast, station T1S1 and T2S4 display the lowest polychaete diversity, each hosting only 2 species. The species belonging to Glyceridae family were found in all stations except T2S4. T1S2 and Station T2S2 inhabit all 4 of the species belonging to Onuphidae family. Only 2 species were found in T1S1, which both are from the Glyceridae family. The complete list of species found in each transect and station are shown in Table 4.1.

Table 4.1: Species composition across the stations in Buntal beach (+ : present; - : absent)

Family	Genus	Species	n	Transect 1				Transect 2			
				S1	S2	S3	S4	S1	S2	S3	S4
Eulepethidae	<i>Buntalepis</i> gen. nov.	<i>Buntalepis</i> <i>sarawakensis</i> sp. nov.	26	-	+	+	-	-	+	+	+
Glyceridae	<i>Glycera</i>	<i>Glycera</i> <i>tridactyla</i>	9	+	+	+	-	+	+	+	-
		<i>Glycera alba</i>	19	-	+	-	+	+	+	+	-
		<i>Glycera</i> <i>tesselata</i>	11	+	+	+	-	+	+	+	-
Nephtyidae	<i>Nephtyidae</i>	<i>Micronephthys</i> <i>sphaerocirrata</i>	7	-	-	+	-	-	+	+	-
Onuphiidae	<i>Onuphis</i>	<i>Onuphis</i> <i>eremita</i>	19	-	+	+	-	-	+	+	-
		<i>Onuphis</i> sp. 1	7	-	+	-	-	-	+	-	-
		<i>Onuphis</i> sp. 2	5	-	+	-	-	-	+	+	-
Sabellidae	<i>Sabellid</i>	<i>Euchone</i> sp. 1	10	-	-	+	+	-	-	+	-
Spionidae	<i>Scoelepis</i>	<i>Scoelepis</i> (<i>Scoelepis</i>) <i>squamata</i>	3	-	-	-	+	-	-	-	+

Among the 10 species collected in this study, 7 were identified to the species level, while 3 were identified to the genus level. A new species, *Buntalepis sarawakensis* **sp. nov.**, was discovered. Only 4 of the identified species (*Glycera tridactyla*, *Glycera alba*, *Glycera tessellata*, and *Micronephthys sphaerocirrata*) had previous records in Malaysia. Among these, only *Micronephthys sphaerocirrata* was recorded in Sarawak. The records in Sarawak and Malaysia waters of other species identified to the genus level were undetermined. Table 4.2 below shows the list of recorded and newly identified polychaete species in Malaysia and Sarawak.

Table 4.2: List of new polychaete species and new records of polychaete species in Sarawak and Malaysia waters (+ : new records; - : recorded; * : undetermined)

Family	Genus	Species	n	New records in Sarawak	New records in Malaysia	New species
Eulepethidae	<i>Buntalepis</i> gen. nov.	<i>Buntalepis sarawakensis</i> sp. nov.	26	+	+	+
Glyceridae	<i>Glycera</i>	<i>Glycera tridactyla</i>	9	+	-	-
		<i>Glycera alba</i>	19	+	-	-
		<i>Glycera tessellata</i>	11	+	-	-
Nephtyidae	<i>Nephtyidae</i>	<i>Micronephthys sphaerocirrata</i>	7	-	-	-
Onuphidae	<i>Onuphis</i>	<i>Onuphis eremita</i>	19	+	+	-
		<i>Onuphis</i> sp. 1	7	*	*	*
		<i>Onuphis</i> sp. 2	5	*	*	*
Sabellidae	<i>Sabellid</i>	<i>Euchone</i> sp. 1	10	*	*	*
Spionidae	<i>Scoelepis</i>	<i>Scoelepis</i> (<i>Scoelepis</i>) <i>squamata</i>	3	+	+	-

4.5.2 Sediment Type of Site Selection

Most stations were dominated by fine sand/silt (63 μm) sediment. Transect 1 showed varying compositions of fine sand/silt (63 μm) and fine sand (125 μm) among stations, while Transect 2 stations consistently had fine sand/silt (63 μm). Overall, sediment at most stations had a high percentage of very fine sand/silt (63 μm) and a low percentage of gravel/coarse sand (1 mm and 500 μm). The biggest percentage of very fine sand/silt was found at T2S4. T1S3 was unique in having a higher percentage of fine sand (125 μm) than very fine sand/silt (63 μm). Sediment types of Buntal beach stations are represented in Figure 4.2.

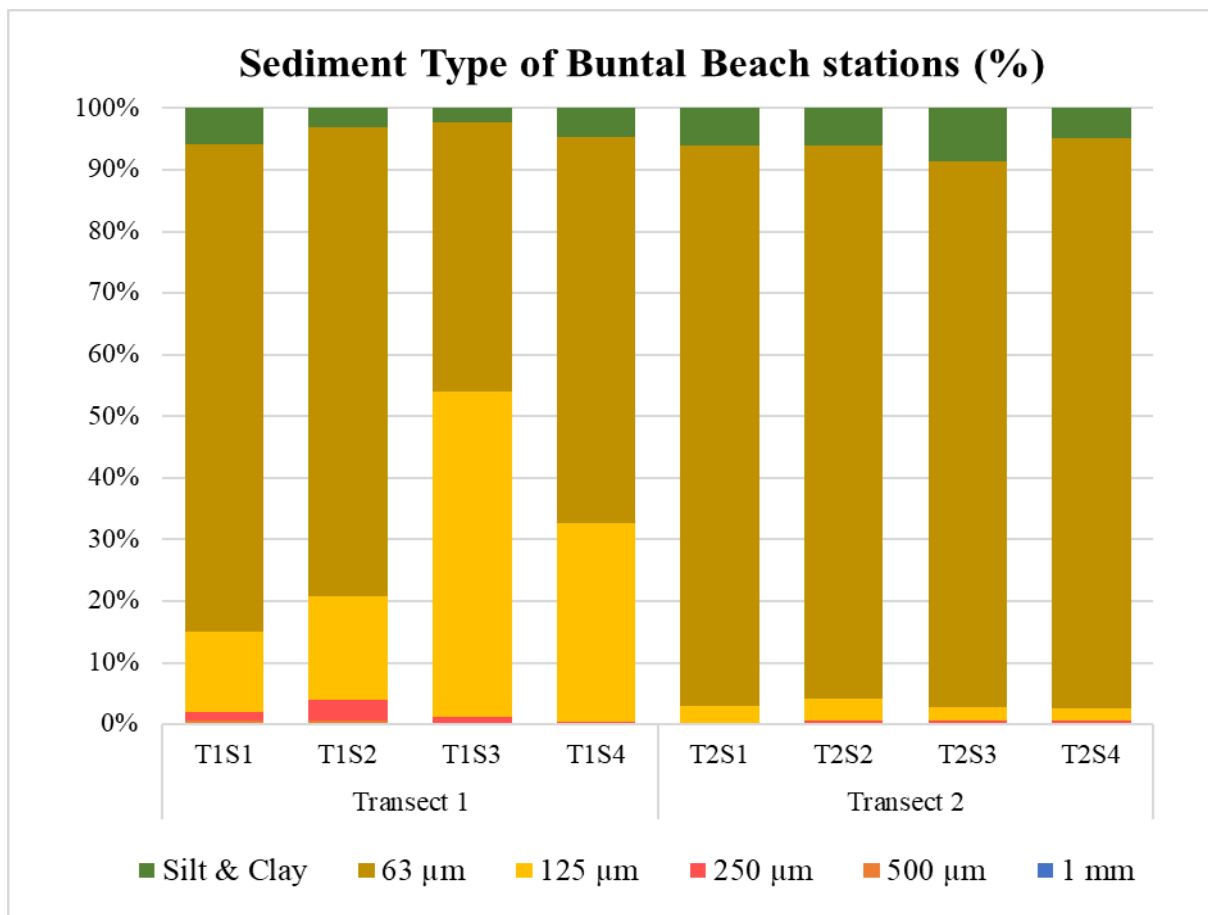


Figure 4.2: Proportion of sediment type on all stations from Buntal Beach (recorded by weight in %)

4.5.3 Key to Polychaete Family of the Buntal Beach, Sarawak

1. A. Dorsal cirri, antennae and acicular/sub-acicular seta present.....2
 B. Dorsal cirri, antennae and acicular/sub-acicular seta absent 5

2. A. Elytra covering upper body, fleshy branchiae replaced by posterior lamellae
 towards the end of body, papillous eversible pharynx end.....Eulepethidae
 B. No elytra but branched form of branchiae present at parapodia.....3

3. A. No eversible proboscis but distinct prostomium with 5 antennae, ceratophores,
 tentacular cirri and pseudocompound hook present Onuphidae
 B. Eversible proboscis, no tentacular cirri and pseudocompound hook..... 4

4. A. Distinct conical head, chitinous jaws may appear on distal end of proboscis, with
 acicular notosetae on parapodia but no lamellae present.....Glyceridae
 B. Indistinct head with ladderred sub-acicular capillaries, vestigial pre-setal and post-
 setal lamellae..... Nephtyidae

5. A. Indistinct prostomium with branchial crown on apex of body with no
 lamellae.....Sabellidae
 B. Conical prostomium with palp covered with sheath, branchiae is thick and
 glandular, notopodial postchaetal lamellae rounded..... Spionidae

4.5.4 Species Descriptions

4.5.4.1 Family Eulepethidae Chamberlin, 1919

Key to the Genus of *Buntalepis* gen. nov. of Buntal Beach

12 pairs of elytra with stouted lateral borders fimbriae, dorsal cirri on segment 3 and 6, 12 pairs of branchia on segment 8, 10, 12...22, 23, 25, 26, posterior lamellae begin on segment 27, presence of two pectinate chaetae in dorsal-most chaetae on 4th parapodia, presence of a single papillae transitioning to capillary towards the end at the centre of the neuropodium lobe..... *Buntalepis* **gen. nov.**

Buntalepis sarawakensis sp. nov.

Genus *Buntalepis* gen. nov.

(Figure 4.3 – Figure 4.10)

Material Examined. Holotype: Malaysia: East Malaysia: Sarawak: Bornean water: Buntal beach, Kuching: 1°42'40' N, 110°22'35" E, 20 March 2021, 1 holotype (catalogue no.: ASD 04258); 1 km off Buntal coast. 26 specimens of this species collected in Buntal coast. Out of 26 specimens, only 1 complete specimen for holotype and 5 complete specimens for paratypes were chosen for detailed morphological description.

Paratypes (5 specimens): Malaysia: East Malaysia: Sarawak: Bornean water: Buntal beach, Kuching: 1°42'05' N, 110°22'34" E, 20 March 2021, 5 paratype (catalogue no.: ASD 04259, ASD 04260, ASD 04261, ASD 04262 & ASD 04263); 1 km off Buntal coast.

Sample diagnosis: Body elongates, size range of material examined 6 – 15 mm long (n= 22), 2.5 mm – 7 mm wide including chaetae, complete specimens with 29 to 34 segments. Anterior part with 12 pairs of elytra on segments, 2, 4, 5, 7, 9, 11, 13, 15, 17, 19, 21, 24, each pair overlapping dorsally, with lateral margins having papillae or 10 – 12 fimbriae stout processes. First pair of elytrae with 9 to 17 papillae on the anterior margin. Elytra 2 – 12 with distinct lateral fimbriae stout projections between the anterior and posterior surfaces. Elytron 2 with 3 – 5 lateral lobes. Elytron 3 with 5 – 8 lateral lobes. Elytron 4, 5, 6, with 7-8 lateral lobes. Elytron 7 with 7 – 9 lateral projections. Elytron 8, 9, 10, with 8 – 10 lateral projections. Elytron 11 with 9 – 12 lateral projections. Elytron 12 with 13 – 14 lateral projections. Lateral projections of elytra elongated digitiform and fimbriae stout. A blunt acicular chaeta on neuropodia of segment 3. Dorsal cirri on segments 3 and 6. Branchiae 12 pairs, on segments 8, 10, alternate segments to 22, 23, 25, 26. Posterior segments beginning on segment 27 each with a pair of posterior lamellae dorsally.

Branchiae 12 pairs, on segments 8, 10, 12, 14, 16, 18, 20, 22, 23, 25, 26 as defined by the presence of distally-branched branchial cirrus, replaced by posterior lamellae on the 27th and the following segments. The lamellae reducing in size towards the posterior but increasing in length and forming a more distinct leaf-shaped distal end towards the back. The branchiae project from the dorsal region of the parapodia and have ventral ciliated bands, along with distally-branched cirrus.

Prostomium is covered by the 2nd segment, antenna and palps reduced and eyes absent if the pharynx is not extended. Median antennae small and shield-shaped, attached to the prostomium on the dorsal margin. Lateral antennae conical and attach laterally relative to the median antenna. Palps are bigger and longer in size compared to the median and lateral

antennae. Palps are elongated and conical in shape, attached behind the median and lateral antennae but laterally relative to the first parapodia.

Parapodia biramous, each ramus supported by aciculum, notoaciculum of median and posterior segments with hooked tip, neuroaciculum throughout expanded distally to form hammer-shaped lobe. Dorsal-most notochaetae curved with the distal edge serrated and rounded, spoon-shaped tip. Ventral notochaetae are robust, slightly bent, and form thin distal end. Notopodial capillaries densely projected on the lower half of the notopodium, projecting from a fleshy lump on the lower notopodial lobe. Notopodial capillaries are also curved, serrated, and formed a sharp tip at the end. There are two forms of the capillaries, 1) entirely smooth and tapering to a fine point, 2) serrated on one side starting from the middle margin continuing for a third of the length of the capillaries.

Dorsal-most neurochaetae are pectinate, ventral neurochaetae consists of smooth capillaries that tapers to a fine tip, capillary chaetae become thinner in more ventral positions. The dorsal neurochaeta of middle parapodia are robust, curved with thin termination at the distal end. The middle neurochaeta of middle parapodia are robust, curved, spiny with a flattened, spatulated distal end. The presence of a neurochaetae on the centre of the neuropodium lobe, laterally positioned from the middle neurochaetae. It is isolated from the alignments of other neurochaetae and exists in a single papilla, smooth and conical, later increases in length and thinner in size and eventually becomes a capillary towards the end body.

On posterior segment, lower notochaete and neurochaetae are much stouter, notochaete are sharply bent, serrated on the upper half of the margin and taper evenly to a rounded tip, neurochaetae are longer, slightly bent at the end, and narrow to a point. Dorsal

cirri present on posterior side of upper margin of parapodia 3 and 6. Ventral cirri on segments 2 – 7 slender, and taper to a tip; on segment 8 and following segments ovoid, articulated, with a minute tip. Posterior lamellae exist starting from 37th segment and continuing posteriorly. Pygidium with right cirrus long, minutely papillated on one side except the basal margin, left cirrus is short and stouted into a blunt tip. The comparison of *Buntalepis* **gen. nov.** with other Eulepethidae genus are displayed in Table 4.3.

Description: The holotype is a complete specimen with 30 segments, 13.0 mm in length and 4.0 mm wide on the median part of the body. Anterior part with 12 pairs of elytra on segments 2, becoming more elongate posteriorly, each pair overlapping dorsally, some overlapping at inner lateral margins of the parallel pair and outer lateral border having thumb-shaped processes between anterior and posterior lobes. First pair elytra oval-shaped, with 9 – 11 papillae on anterior borders. The remaining elytra sub rectangular, elongated posteriorly approaching towards the end of the body, with 5 – 13 processes or articulated digitiforms. In holotype first left elytra, dorsal margin with 10 digitiforms. For elytra 2 and 9, lateral margin with 5 articulated thumb-shaped processes. Elytra 3 with 6 articulated thumb-shaped processes. Elytra 4 and 6 with 8 articulated thumb-shaped processes. Elytra 5, 7, 8 and 10 with 7 articulated thumb-shaped processes. Elytra 9 with 5 articulated thumb-shaped processes. Elytra 11 with 9 articulated thumb-shaped processes and elytra 12 with 14 articulated thumb-shaped processes. Parapodia is biramous. Neurochaetae of several kinds: 1 – 2 upper pectinate chaetae; flattened chaetae with long, tapered tips on anterior parapodia; simple chaetae with pseudo-articulation; presence of a single papillae on the centre of the neurochaetae; lower neurochaetae with rows of spinules; chaetae with tapering tips; acicular neurochaetae on segment 3. Posterior lamellae on 37th segment and continuing posteriorly. Pygidium with anal cirrus.

Distribution of Eulepethidae: Australia, Papua New Guinea, Indonesia, Riau Islands, Andaman Sea, Sri Lanka, Taiwan, Hong Kong, Red Sea, Madagascar, Gulf of Mexico, Peninsular Malaysia; in this study *B. sarawakensis* **sp. nov.** was found in 5 stations, T1S2, T1S3, T2S2, T2S3, T2S4, which shows very fine sand to medium sand type of sediment.

Type Locality: *B. sarawakensis* **sp. nov.** currently only occur in Buntal Bay, Kuching, East Malaysia: Sarawak: Bornean water: 1°42'05' N, 110°22'34" E; 1°42'17' N, 110°22'43" E. living on the sandy and silty sand area of Buntal intertidal. This is the first record of family Eulepethidae in Sarawak and genus *Buntalepis* **gen. nov.** in Malaysia.

Etymology: The specific name *Buntalepis* is derived from Buntal beach, the type locality in Sarawak, while *sarawakensis* is derived from the name 'Sarawak'.

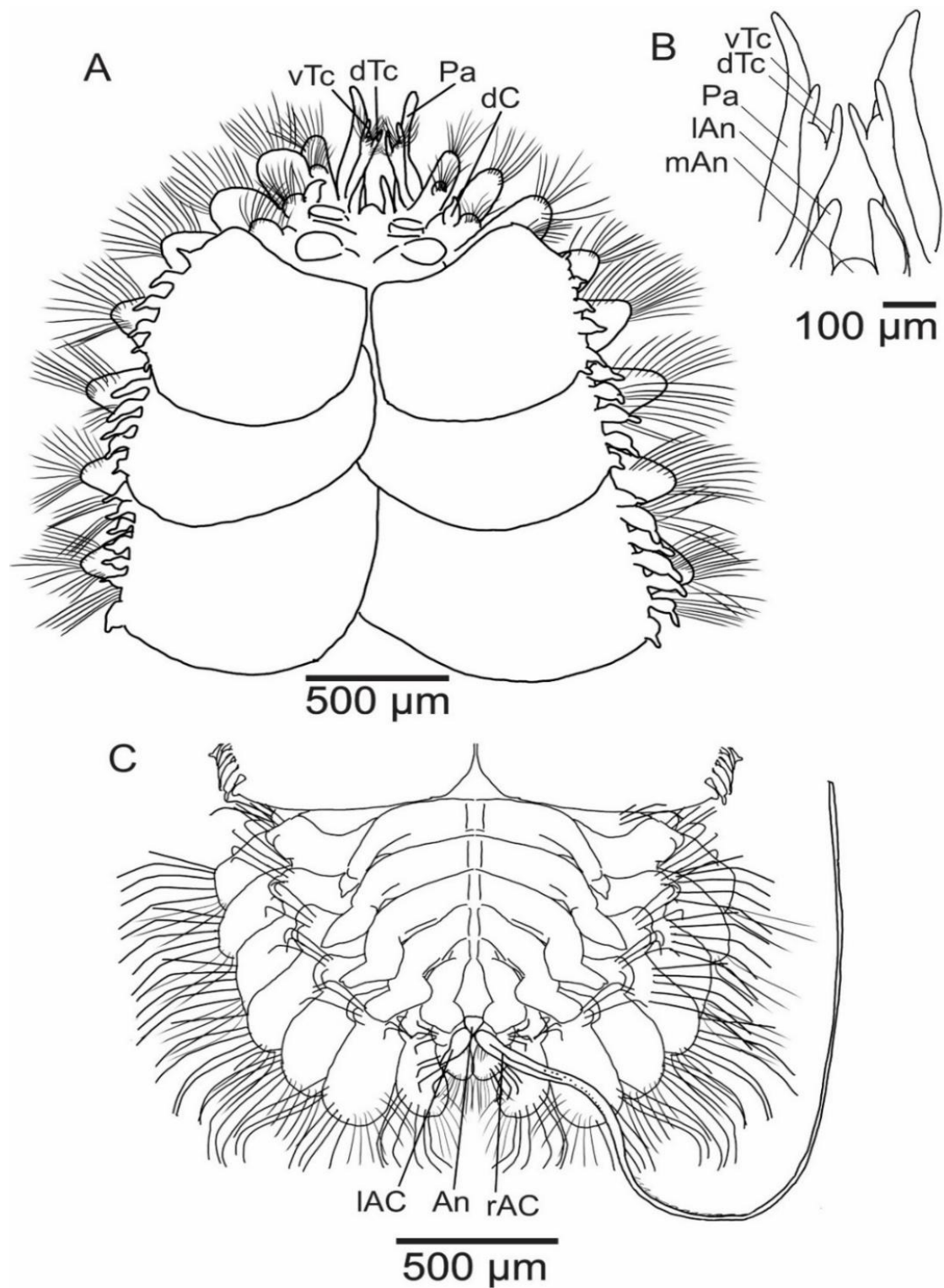


Figure 4.3: *Buntalepis sarawakensis* sp. nov., holotype ASD 04258, A, anterior region, dorsal view, 1st and 2nd elytron removed; B, dorsal view of prostomium; C, dorsal view of pygidium and posterior segments, left and right cirrus present.

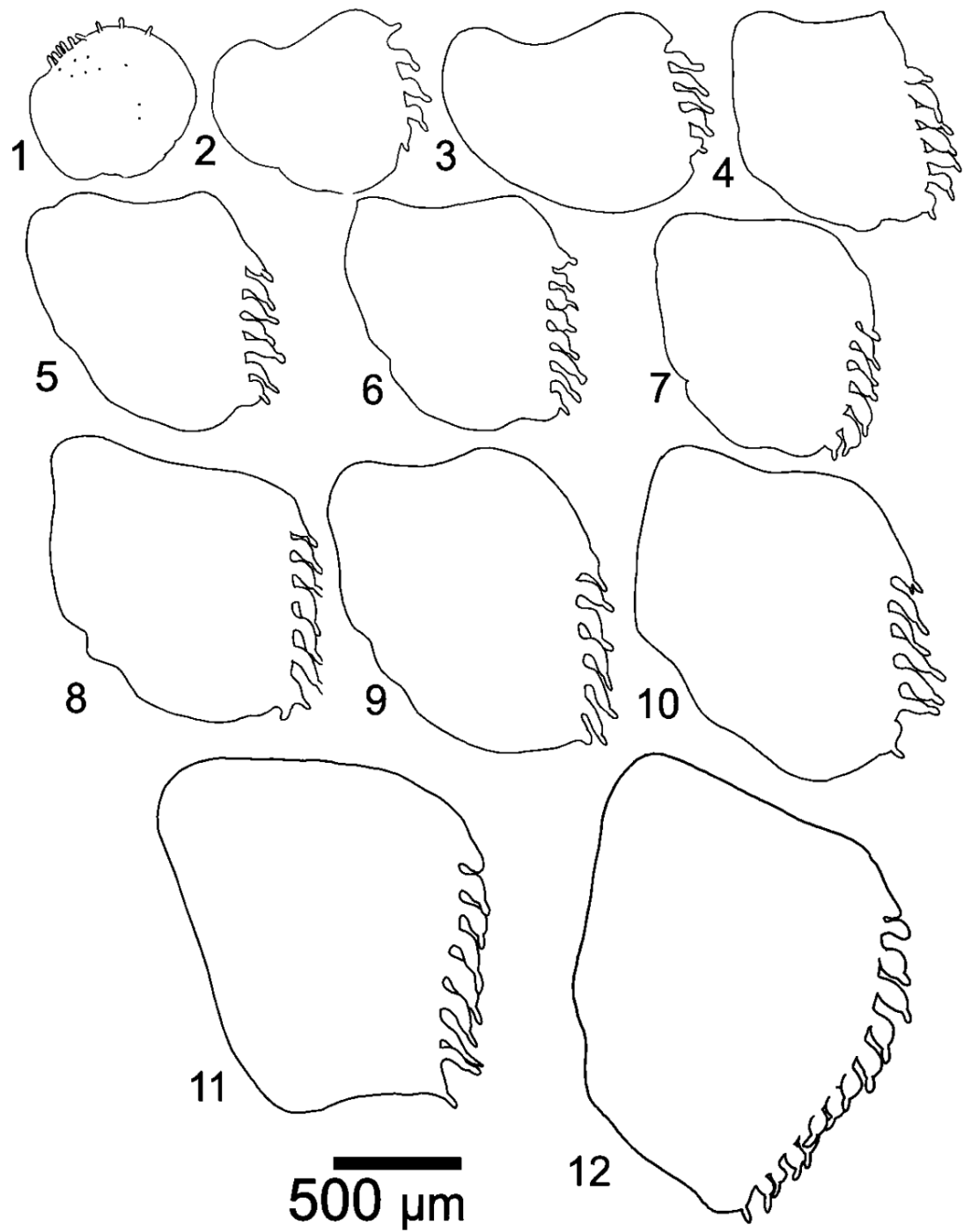


Figure 4.4: *Buntalepis sarawakensis* **sp. nov.**, holotype ASD 04258, 1st – 12th right elytron.

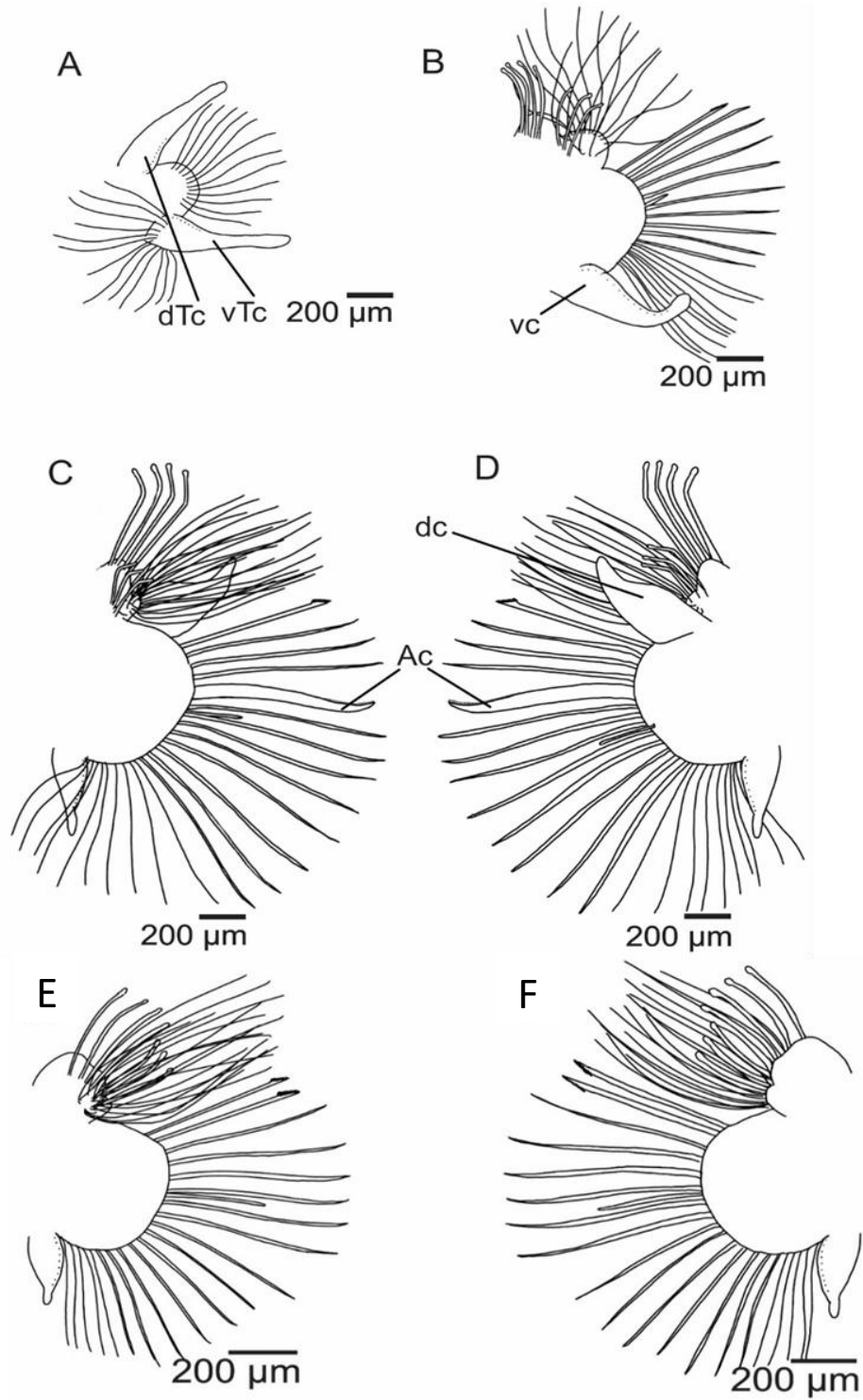


Figure 4.5: *Buntalepis sarawakensis* sp. nov., holotype ASD 04258, A, dorsal view of left tentacular parapodium, segment 1; B, posterior view of right parapodium, segment 2; C, posterior view of right parapodium, segment 3; D, anterior view of right parapodium, segment 3; E, posterior view of right parapodium, segment 4; F, anterior view of right parapodium, segment 4.

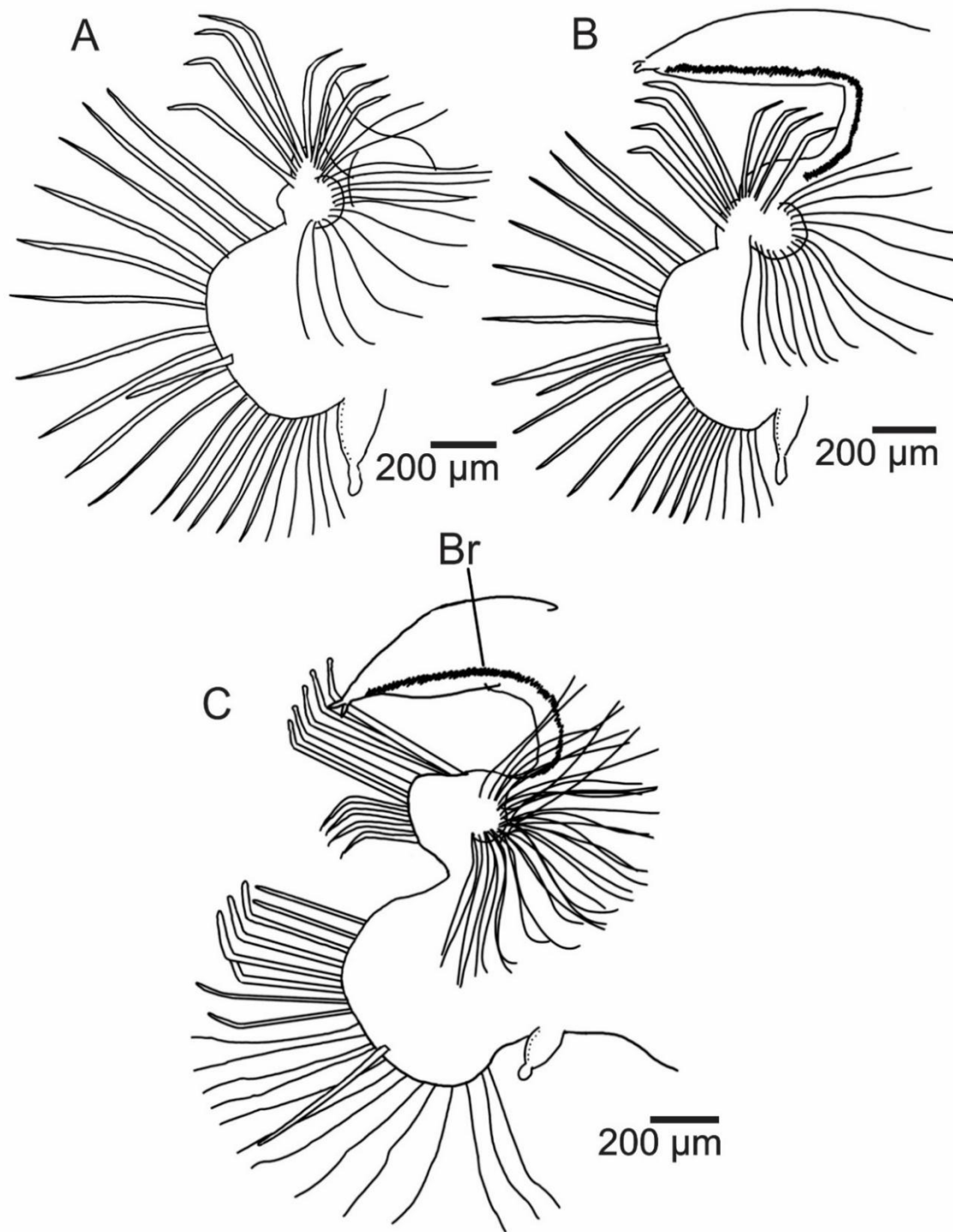


Figure 4.6: *Buntalepis sarawakensis* **sp. nov.**, holotype ASD 04258, A, anterior view of right parapodium, segment 6; B, anterior view of right parapodium, segment 8; C, anterior view of right parapodium, segment 22.

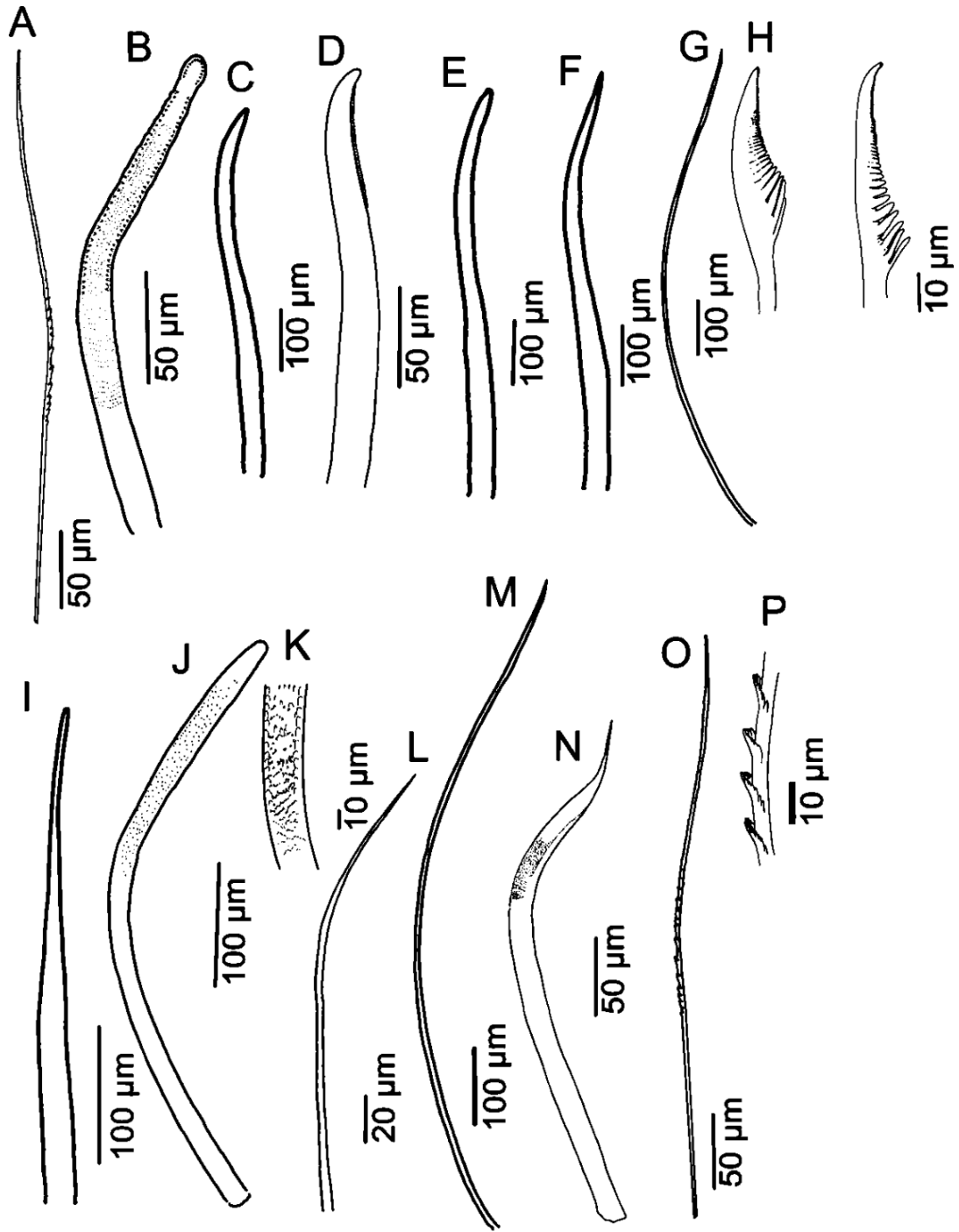


Figure 4.7: *Buntalepis sarawakensis* sp. nov., holotype ASD 04258, A, capillary notochaeta from segment 1; B – H, chaetae from segment 3; B, hooked notochaeta; C, dorsal neurochaeta; D, serrated acicular neurochaeta; E, middle neurochaeta; F, ventral neurochaeta; G, capillary neurochaeta; H, pectinate neurochaeta; I – O, chaete from segment 22; I, dorsal neurochaeta; J, middle neurochaeta; K, upper margin of middle neurochaeta; L, ventral neurochaeta; M, capillary neurochaeta; N, ventral hooked notochaeta; O, capillary notochaeta; P, serrated margin of capillary notochaeta

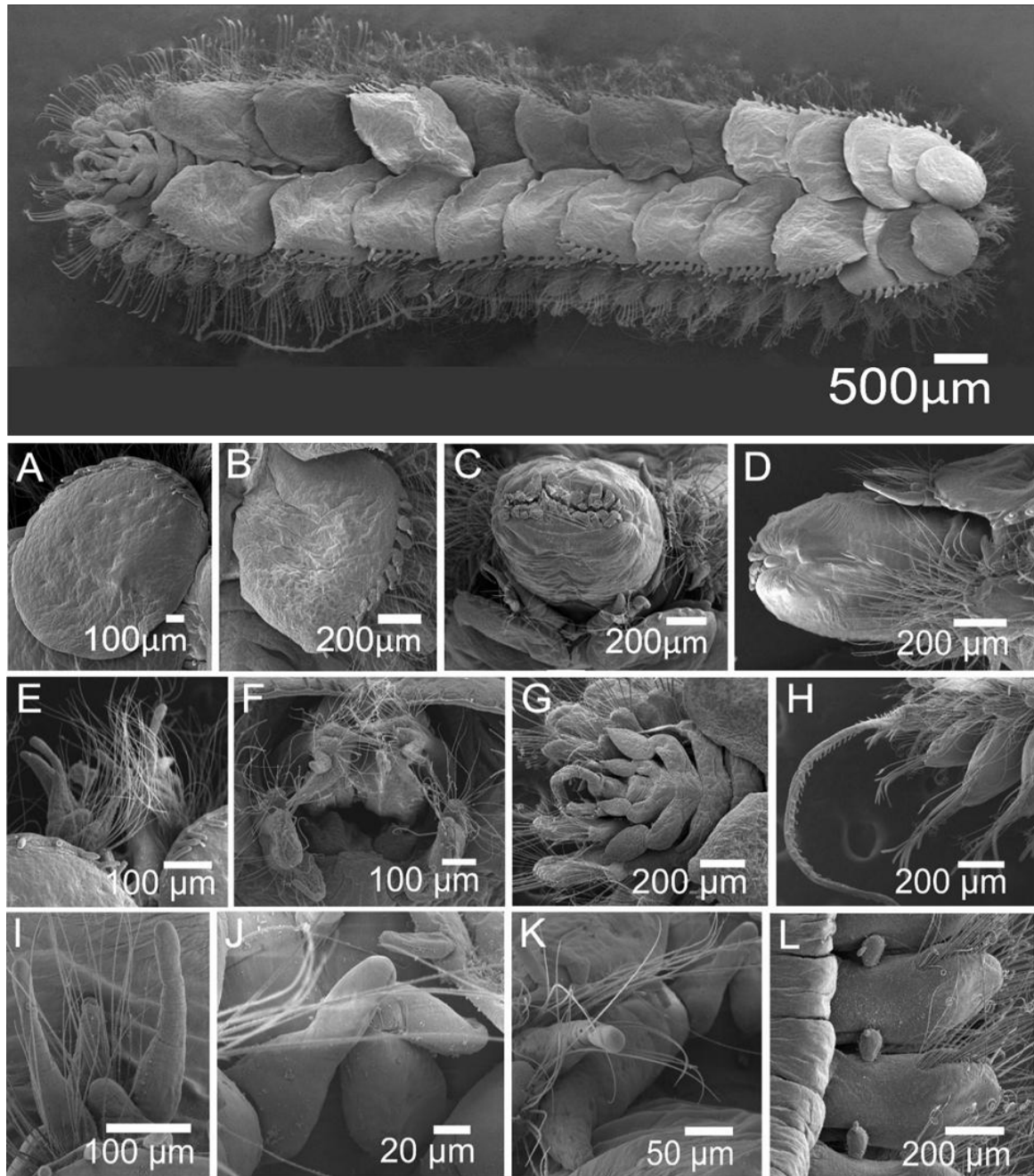


Figure 4.8: *Buntalepis sarawakensis* **sp. nov.**, holotype ASD 04258, Entire worm; A1, dorsal view of the entire worm; A, 1st left elytra; B, 12th right elytra; C, anterior view of extended pharynx; D, distal right half of pharynx, inner view; E & F, dorsal and anterior view of anterior end; G & H, pygidium and final posterior segments, dorsal and ventral view; I, 1st parapodia; J, median antennae; K, palp; L, dorsal view of the middle parapodia, dorsal cirri.

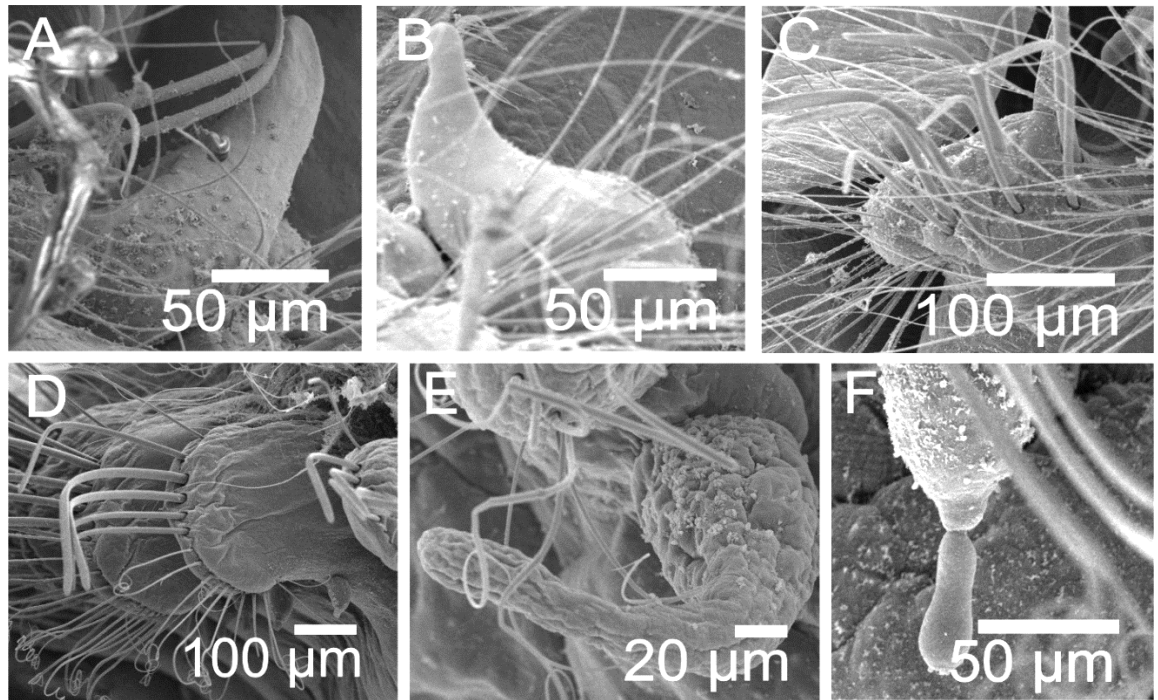


Figure 4.9: *Buntalepis sarawakensis* **sp. nov.**, holotype ASD 04258, A, dorsal cirri of 3rd segment; B, dorsal cirri of 6th segment; C, notopodium with notochaetae attached; D, hammer-shaped neuropodium with neurochaetae attached; E, ventral cirri of 1st parapodia; F, ventral cirri of 7th parapodia.

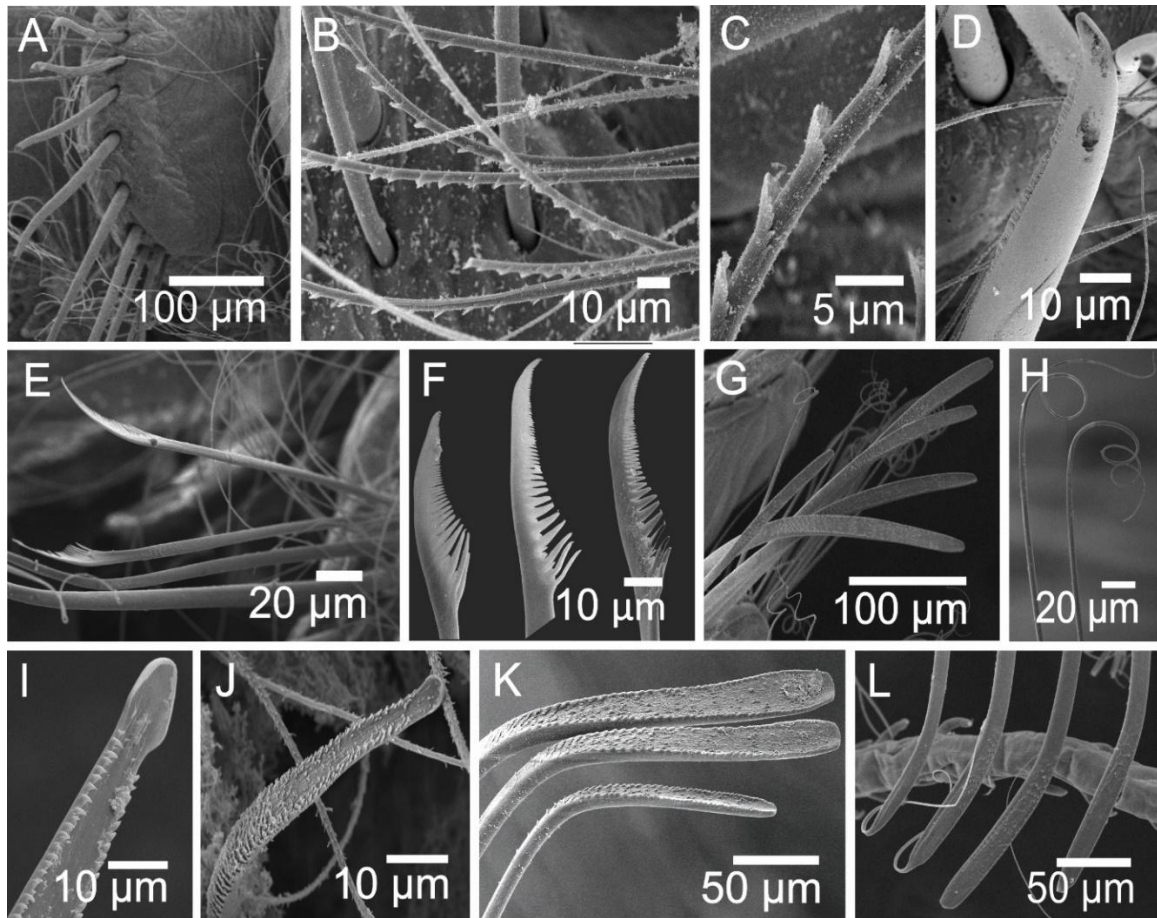


Figure 4.10: *Buntalepis sarawakensis* **sp. nov.**, holotype ASD 04258, A, middle neurochaetae, slightly bent at the end and narrow to a point; B & C, dorsal-most notochaetae, serrated capillaries; D, serrated acicular neurochaetae on 3rd parapodia; E, dorsal-most neurochaetae, pectinate chaetae on 4th segment; F, pectinate chaetae on 5th, 10th and 25th segment; G, slightly bent, serrated on the upper half of the margin and taper evenly to a rounded tip on 26th segment; H, smooth capillaries tapering to a fine point on 14th segment; I & J, hooked notochaetae with serrated body margin and disciform tip on 3rd segment; K, neurochaetae on 30th segment, slightly bent, serrated on the upper half of the margin and tapered evenly to a rounded tip; L, neurochaetae on 24th segment, longer, slightly bent at the end, narrow to a point.

Table 4.3: Comparison of *Buntalepis* **gen. nov.** with other Eulepethidae genera (based on previous studies).

Genus/Trait	<i>Eulepethus</i> (Chamberlin, 1919)	<i>Grubeulepis</i> (Pettibone, 1969)	<i>Mexieulepis</i> (Rioja, 1962)	<i>Pareulepis</i> (Darboux, 1900)	<i>Lamelleulepethus</i> (Pettibone, 1986)	<i>Proeulepethus</i> (Pettibone, 1986)	<i>Buntalepis</i> gen. nov.
Body segment	up to 70	about 38 (32-40)	about 50 (37-62)	about 37 (34-38)	about 35	up to 39	Up to 34
Elytra	Numerous pairs	12 pairs	Numerous pairs	12 pairs	12 pairs	12 pairs	12 pairs
Elytra processes	Lateral notch only or leaf-like process inside the notch.	Lateral borders fimbriated.	Lateral margins fimbriated.	Lateral borders notched.	Lateral borders fimbriated.	Lateral borders entire, not notched or fimbriated.	Lateral borders fimbriae stoutened.
Dorsal cirri	On segments 3 & 6	On segments 3 & 6	On segments 3 & 6	On segments 3 & 6	On segments 3 & 6	On segments 3 & 6	On segments 3 & 6
Branchiae	12 pairs, on segments 8, 10...22, 23, 25, 26, 27.	10-13 pairs, on segments 8, 10...22, 23, 25, (26, 27, 28).	11-12 pairs, on segments 8, 10...22, 23, 25, 26 (sometimes 27)	12 pairs, on segments 8, 10...22, 23, 25, 26, 27.	12-13 pairs, on segments 8, 10...22, 23, 25, 26, 27, (28).	10-11 pairs, on segments 8, 10...22, 23, 25, (26).	12 pairs, on segments 8, 10...22, 23, 25, 26.
Posterior lamellae	Not described	Begin on segments 26-29. Treadwell, 1901; Berkeley & Berkeley; 1939; Jones, 1962;	Not described	Begin on segment 28.	Begin on segment 28 or 29,	Begin on segments 26-27,	Begin on segment 27.
References	Grube, 1875; Zhang <i>et al.</i> , 2017	Imajima, 1974; Berkeley & Berkeley, 1939; Horst, 1913; McIntosh, 1885	Imajima, 1974; Berkeley & Berkeley, 1939; Horst, 1922	Horst, 1913; McIntosh, 1885	Pettibone, 1986	Pettibone 1986, McIntosh, 1885; Woolley & Wilson, 2011	This study

4.5.4.2 Family Glyceridae Grube, 1850

Key to the Species of *Glycera* of Buntal Beach

1. Presence of long eversible pharynx and 4 small antennae at the tip of the prostomium, proboscis covered in proboscideal papillae.....2
2. A) No jaw element presents at the tip of the proboscis, proboscideal papillae with short stalks.....3
B) Jaw element presents at the tip of the proboscis, presence of aileron and presence of proboscideal papillae with long stalks.....4
3. Proboscideal papillae with “V”- shaped ridges covering posterior surface, conical papillae with median longitudinal ridge and rounded papillae with a very distinct median longitudinal ridge. Neuropodial postchaetal lobes on early parapodium are digitiform and triangular, but rounded in mid body parapodia and posterior parapodia, compound spinigers are flat with finger-shaped opening on outer side..... *Glycera tridactyla* (Grube, 1861)
4. A) Proboscideal papillae with numerous, conical papillae with leaf- shaped ridges on posterior surface, rounded and conical papillae with smooth surface. Aileron with triangular base. Compound spinigers are thick and rounded, with finger-shaped opening on outer side of the spiniger tip *Glycera alba* (O.F. Müller, 1776)
B) Proboscideal papillae have short stalks and consist of numerous, conical papillae with leaf- shaped ridges covering the posterior surface. Aileron with deeply incised

base. Compound spinigers are thin and stouted, with crown-shaped opening on the spiniger tip..... *Glycera tessellata* (Grube, 1863)

***Glycera tridactyla* (Grube, 1861)**

(Figure 4.11 – Figure 4.17)

Family Glyceridae, Grube, 1850

Genus *Glycera* Lamarck, 1818

Material Examined: Paratype (2 samples): Malaysia: East Malaysia: Sarawak: Bornean water: Buntal beach, Kuching: 1°42'40' N, 110°22'35" E, 20 March 2021; 2 paratypes (catalogue no.: ASD 04264 & ASD 04265). 1 km off Buntal coast. Out of 9 specimens collected in Buntal coast, only 2 complete specimens were chosen as paratypes for detailed morphological description.

Description: The total length of the sample is about 30 mm, with about 120 segments, and has a width of about 3 mm in the middle body. Prostomium long, tapered, tipped with four small biannulate antennae and superficially ringed. Prostomium shaped of a slender cone with about 10 rings. The proboscis is extended and covered with long cylindrical papillae, include forms and numerous very long, slender forms which are not ringed. No jaw element presents at the tip of the proboscis. Proboscical papillae mainly with terminal fingernail structure with short stalk and same longitudinal ridges on nail; ailerons with triangular bases; parapodia of mid body with slender triangular notopodial and shorter, more or less rounded neuropodial postchaetal lobes; simple, digitiform branchiae, situated termino-dorsally on parapodia, from anterior to near posterior end. The papillae consist of three types: (1) numerous, conical papillae with three “V” – shaped ridges on posterior surface from top to bottom; (2) a few long, conical papillae, with one straight, median

longitudinal ridge; and (3) very few, slightly shorter and broader, rounded papillae with a single very distinct median longitudinal ridge. The proboscis is extended and covered with long cylindrical papillae, include forms and numerous very long, some are smooth and slightly oval, most are ringed slender form. The pattern of the proboscideal papillae is low saturation on the lower part, vertically arranged from lower part towards the middle part of the proboscis and heavily saturated from the upper middle towards the tip of proboscis and inner opening of the proboscis. Proboscis opening is horizontally narrow and internally filled with long, cylindrical papillae.

The first two parapodia is uniramous, with a prechaetal and a postchaetal lobe. Parapodia is biramous after 2nd feet. Dorsal cirri start from 2nd feet while ventral cirri start from 3rd feet. Several compound notochaetae on the notopodial lobe found in the 1st and 2nd feet. Neuropodial lobe start from 3rd feet. There are 2 triangular to digitiform, prechaetal and postchaetal anterior lobes, and lobes of similar length in mid body. Pointed-shaped dorsal cirrus from parapodium 2 eventually become digitiform-shaped continuing posteriorly. Ventral cirri are not distinct in anterior part, but are well-developed in the posterior part. Notochaetae slender capillaries with one margin covered with spines or hairs. Compound spinigers are thin and flat, with distally flattened finger-shaped opening on the outer side. Presence of neurochaetae homogomph spinigers and notochaetae slender capillaries with one margin covered with spines or hairs. The spinigers and capillaries starts disappearing at the end of the body. Only the dorsal cirri, prechaetal and postchaetal lobes, and ventral cirri present at final five segments. At the lateral side of the body end, the dorsal cirri, prechaetal and postchaetal lobes, and ventral cirri merges into one long digitiform. A long, pointed and smooth tail-like papillae present at the pygidium of the body.

Distribution: Hong Kong, India, Japan, Borneo, Australia, Thailand, India, Kuwait, France, Italy, Portugal, Spain, United Kingdom, Australia, Papua New Guinea; in this study, *Glycera tridactyla* was found in 6 stations (T1S1, T1S2, T1S3, T2S1, T2S2, T2S3), which shows very fine sand to medium sand type of sediment.

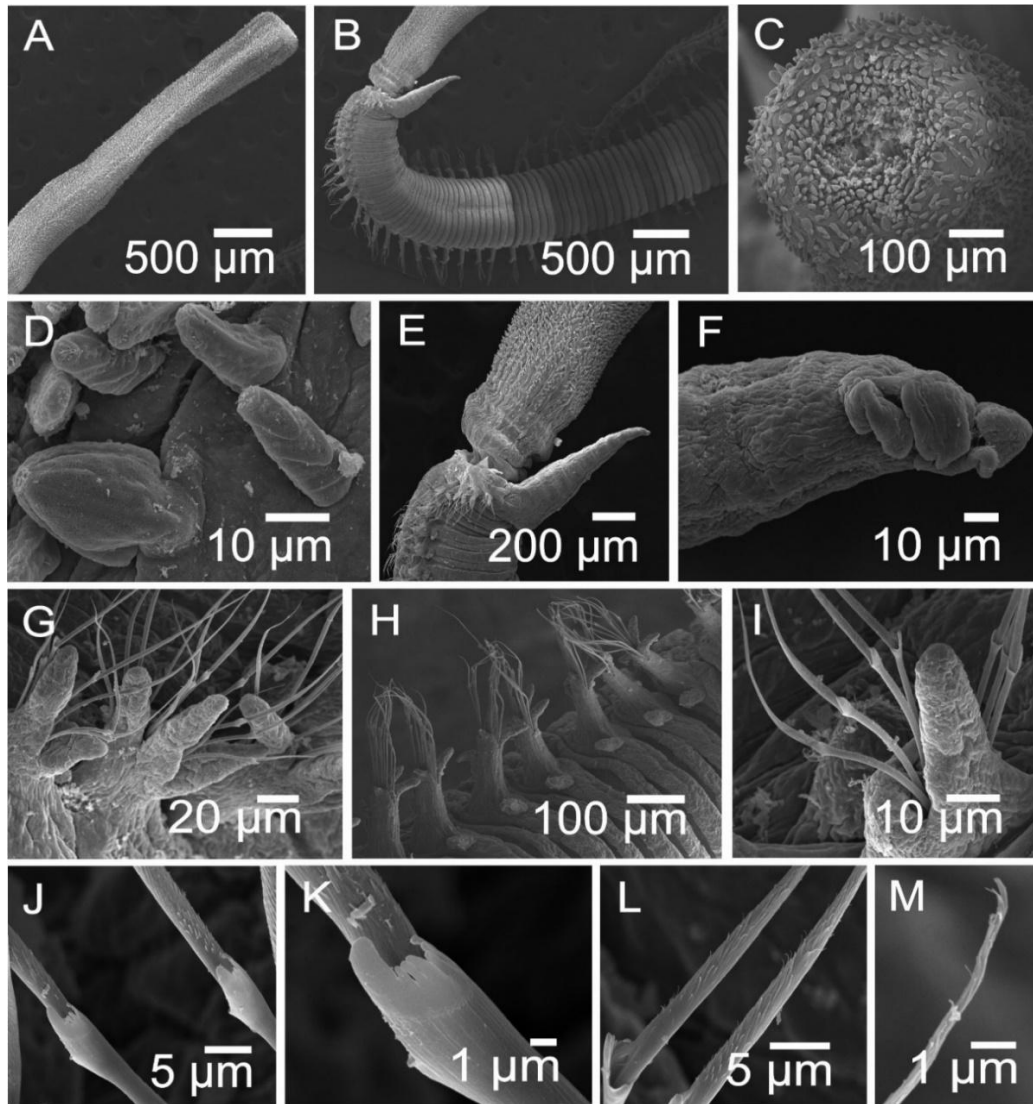


Figure 4.11: *Glycera tridactyla*. A, proboscis, ventral view; B, full body; C, proboscis, dorsal view; D, proboscideal papillae, 3 types; E, proboscis and prostomium; F, prostomium, distal end branched to 4 antennae; G, 1st to 3rd parapodia; H, 20th to 27th parapodia; I, 1st parapodia; J & K, compound spiniger neurochaetae; L, compound spiniger neurochaetae, upper margin; M, compound spiniger neurochaetae, taper to a thin end.

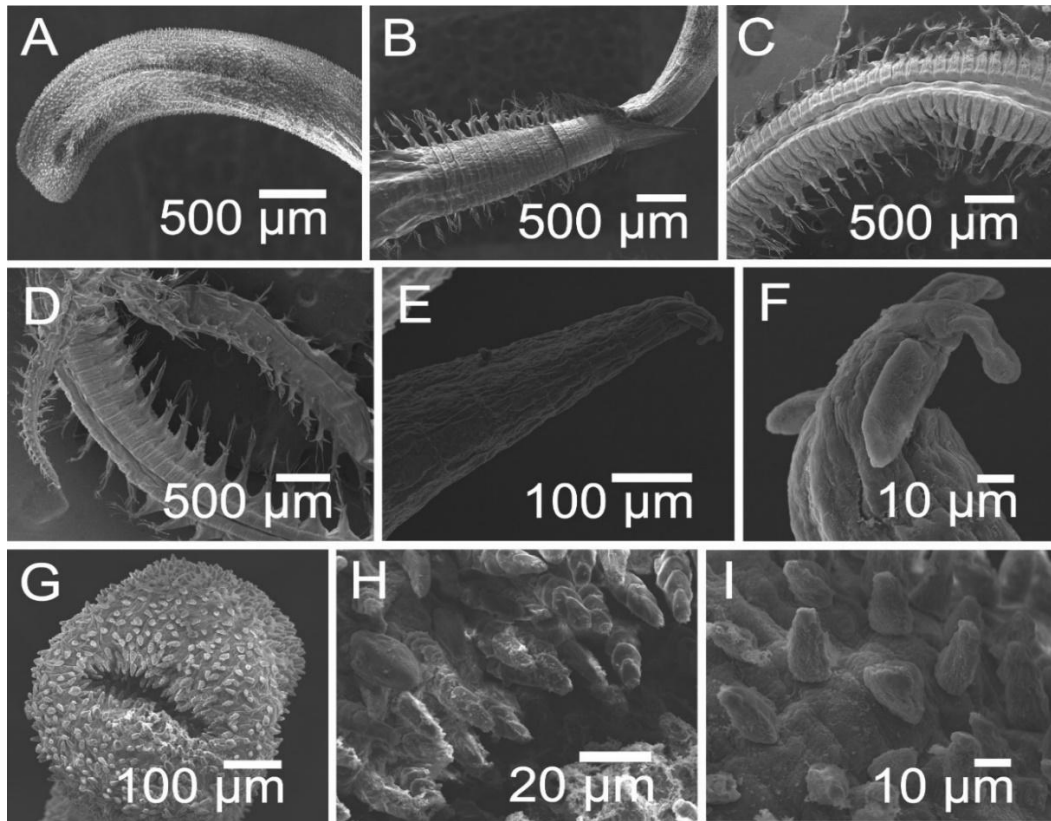


Figure 4.12: *Glycera tridactyla*. A, proboscis, ventral view; B, full body; C, middle body; D, anterior part of body and pygidium; E & F, prostomium, distal end branched to 4 antennae; G, proboscis, dorsal view; H & I, proboscideal papillae.

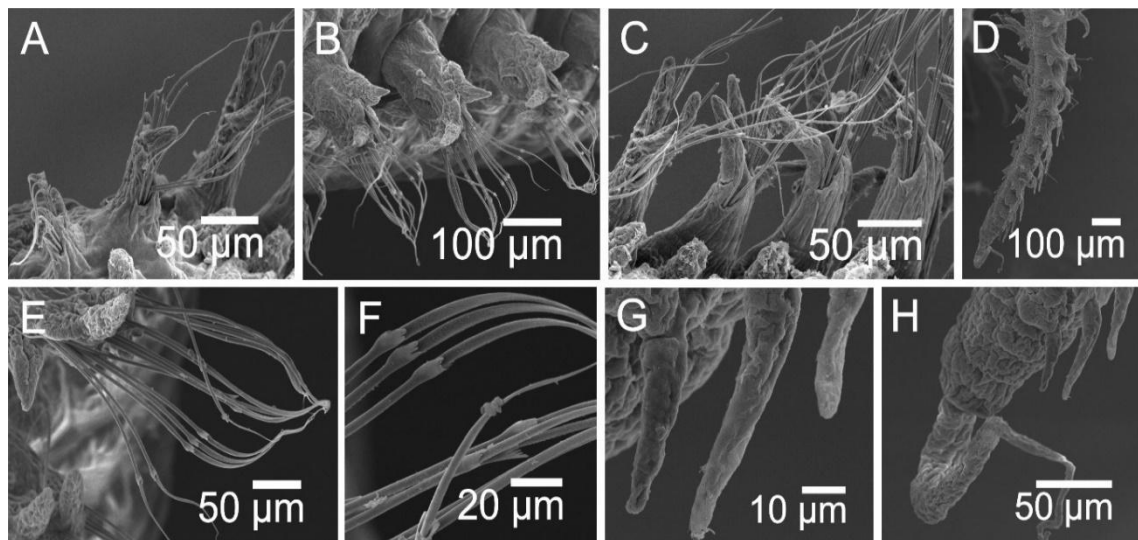


Figure 4.13: *Glycera tridactyla*. A, 1st to 3rd parapodia; B, 4th to 6th parapodia; C, 20th to 24th parapodia; D, posterior part of body; E & F, compound neurochaetae; G, elongated dorsal cirri; H, pygidium.

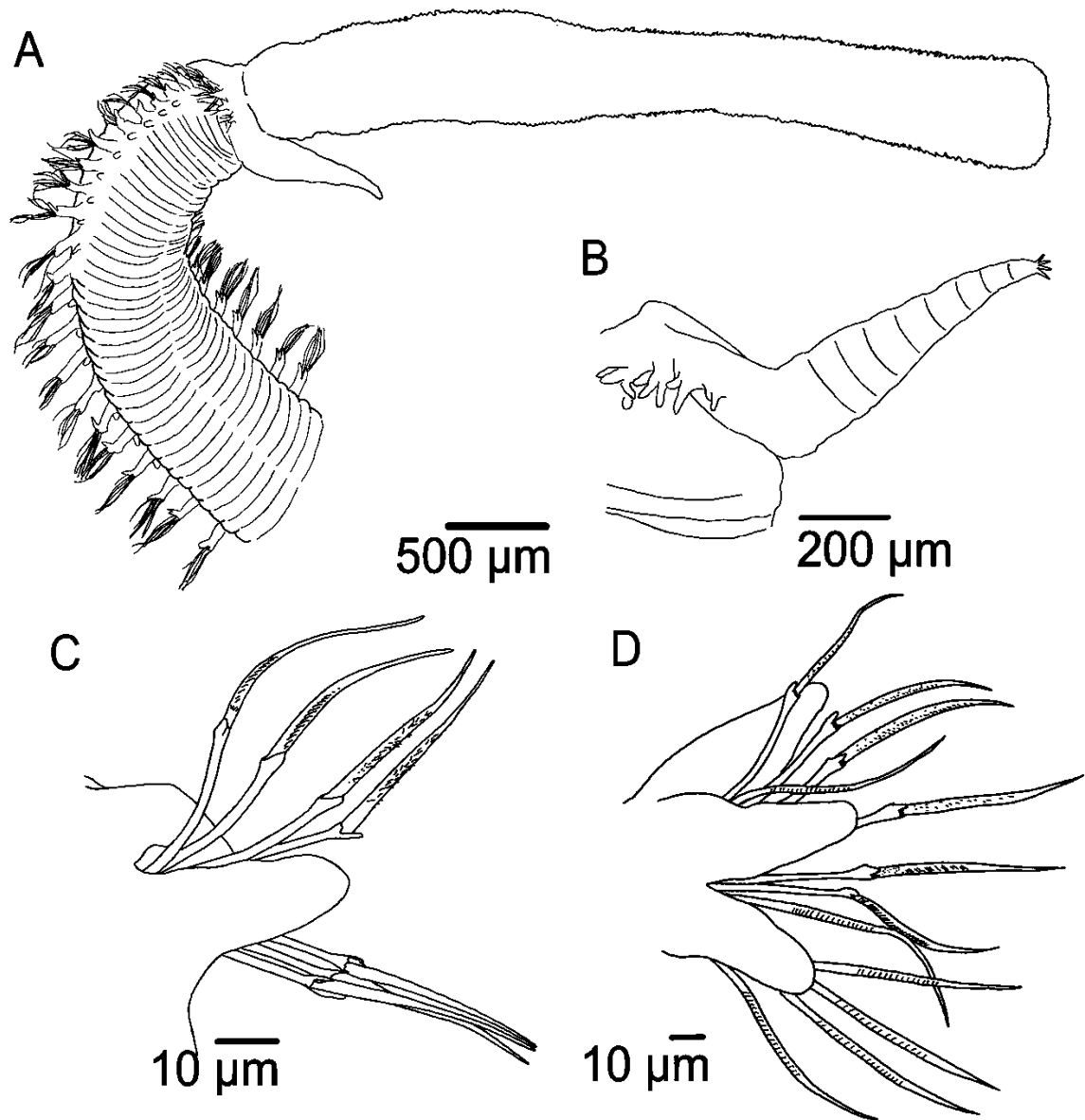


Figure 4.14: *Glycera tridactyla*. A, full body view; B, prostomium / head; C, 1st parapodia; D, 3rd parapodia.

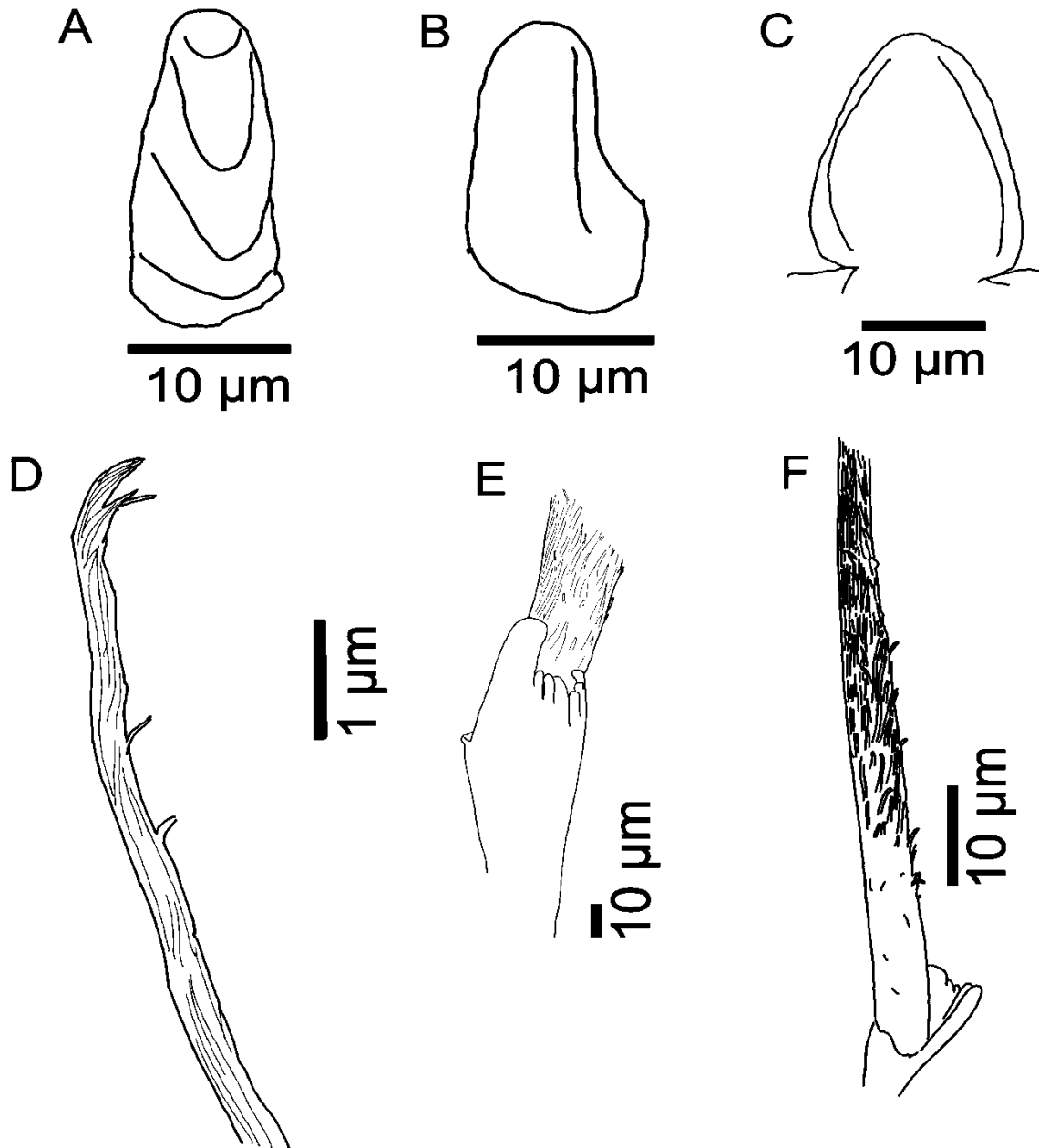


Figure 4.15: *Glycera tridactyla*. A – C, proboscideal papillae, 3 types; A, conical papillae with “V”-shaped ridges on upper surface from top to bottom; B, long, conical papillae, with median longitudinal ridge; C, shorter and broader, rounded papillae with a single very distinct median longitudinal ridges; D, compound spiniger, distal end; E, compound spiniger, middle margin with distally flattened finger-shaped opening on the upper side of the spiniger; F, compound spiniger, whole.

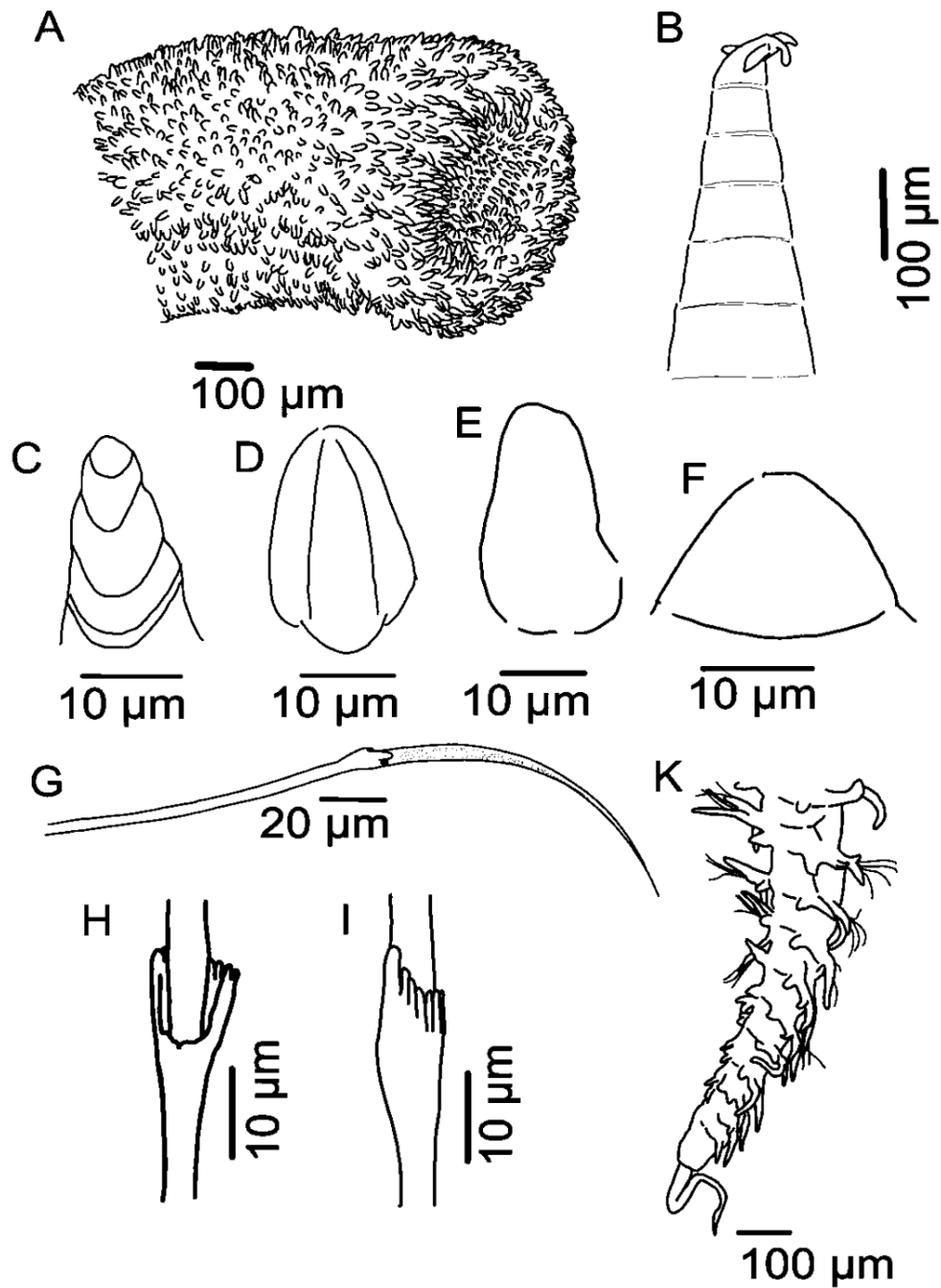


Figure 4.16: *Glycera tridactyla*. A, anterior end of proboscis with proboscideal papillae; B, distal end of prostomium with conical rings and 4 small antennae; C – F, different types of proboscideal papillae; C, conical papillae with “V”-shaped ridges on upper surface from top to bottom; D, long, conical papillae, with one straight, median longitudinal ridge; E, conical papillae with smooth surface; F, shorter and stouter conical papillae with smooth surface; G, compound spiniger, middle margin; K, posterior end of the body; pygidium.

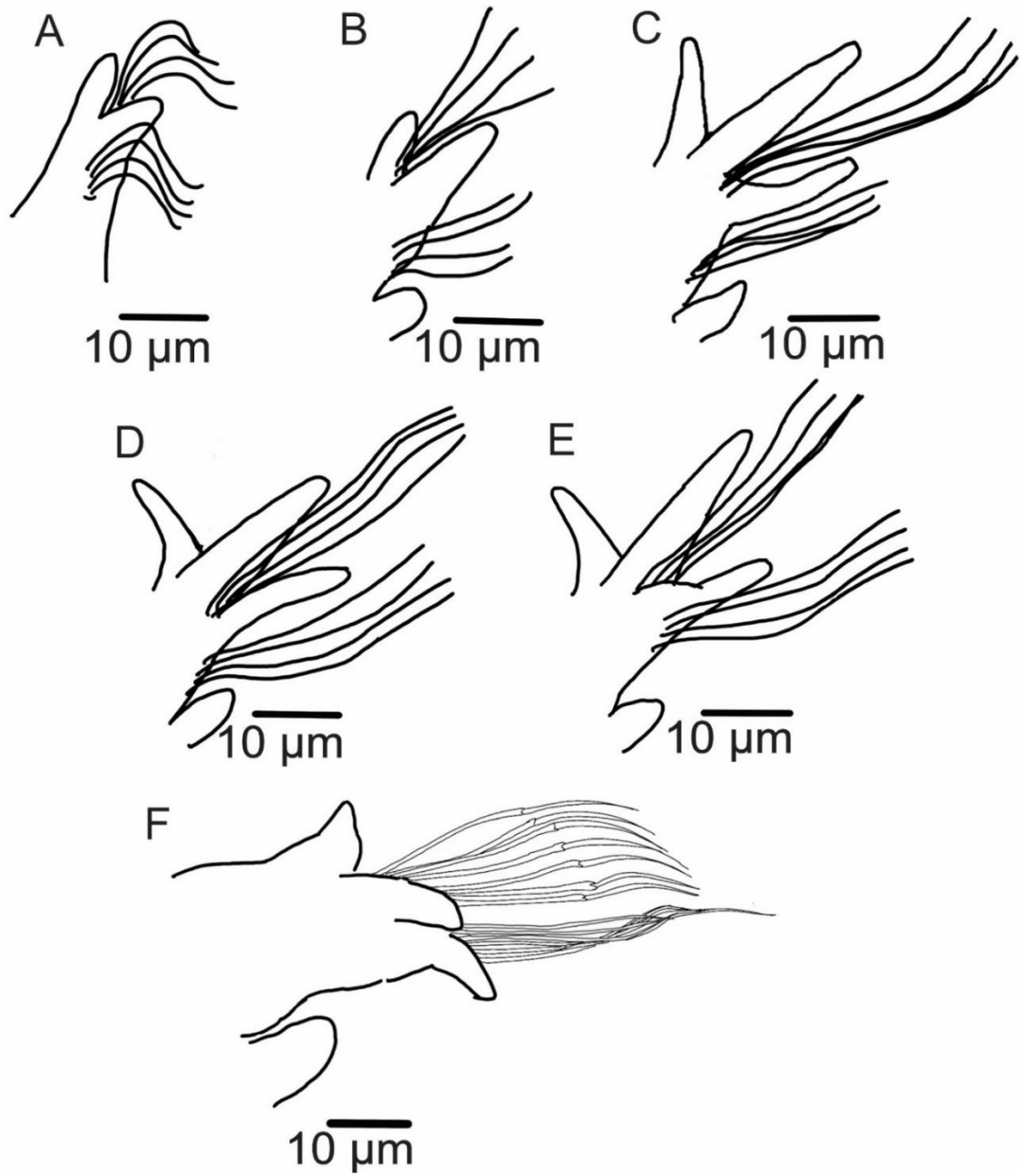


Figure 4.17: *Glycera tridactyla*. A, 1st parapodia; B, 2nd parapodia; C, 3rd parapodia; D, 5th parapodia; E, 6th parapodia; F, middle parapodia.

***Glycera alba* (O. F. Müller, 1776)**

(Figure 4.18 – 4.20)

Family Glyceridae, Grube, 1850

Genus *Glycera* Lamarck, 1818

Material Examined: Paratype (2 samples): Malaysia: East Malaysia: Sarawak: Bornean water: Buntal beach, Kuching: 1°42'40' N, 110°22'35" E, 20 March 2021. 2 paratypes (catalogue no.: ASD 04266 & ASD 04267). 1 km off Buntal coast. Out of 19 specimens collected in Buntal coast, only 2 complete specimens were chosen as paratypes for detailed morphological description.

Description: The whole body reaches up to 35 mm long, with up to 135 segments, has a width of 3 mm in the middle part of the body; preserved specimens in alcohol are pale yellowish. The body segments are biannulate and the anterior annulus shorter than the posterior annulus. The prostomium is conical, pointed and distinctly separated into about 10 – 12 rings; the terminal rings have four antennae. The proboscis is shorter and thicker in general compared to the posterior end of the body. The proboscis is about 9 mm in length and width of the anterior end of proboscis is about 2.4 mm. The proboscis is bell-shaped and densely covered with papillae which are arranged in distinct longitudinal rows. The papillae consist of three types: (1) numerous, conical papillae with leaf-shaped ridges on posterior surface from top to bottom; (2) very few, slightly shorter and broader, rounded papillae with smooth surface; and (3) a few long, conical papillae, with smooth surface. Among the three types, type 3 is the longest. The terminal part of the proboscis has four black hook-shaped jaws with several vertically-arranged incision on its lateral side. The parapodia of the first two segments are uniramous, while the subsequent parapodia are biramous. There are 2

triangular to digitiform, prechaetal and postchaetal anterior lobes, and lobes of similar length in mid body. Compound spinigers are thick and rounded, with distally stout finger-shaped opening on outer side of the spiniger tip. Pointed-shaped dorsal cirrus from parapodium 2 eventually become digitiform-shaped continuing posteriorly. Knob-like dorsal cirri from the second parapodium are inserted most clearly at the base of the anterior parapodia, as well as on the body wall far above parapodial base in the mid body, and again near the posterior base. Ventral cirri are not distinct in anterior part, but are well-developed in the posterior part. Two long, pointed and smooth tail-like papillae present at the pygidium of the body.

Distribution: China, Hong Kong, Vietnam, Iran, Belgium, France, Ireland, Italy; in this study, *Glycera alba* was found in 5 stations (T1S2, T1S4, T2S1, T2S2, T2S3), which shows very fine sand to medium sand type of sediment.

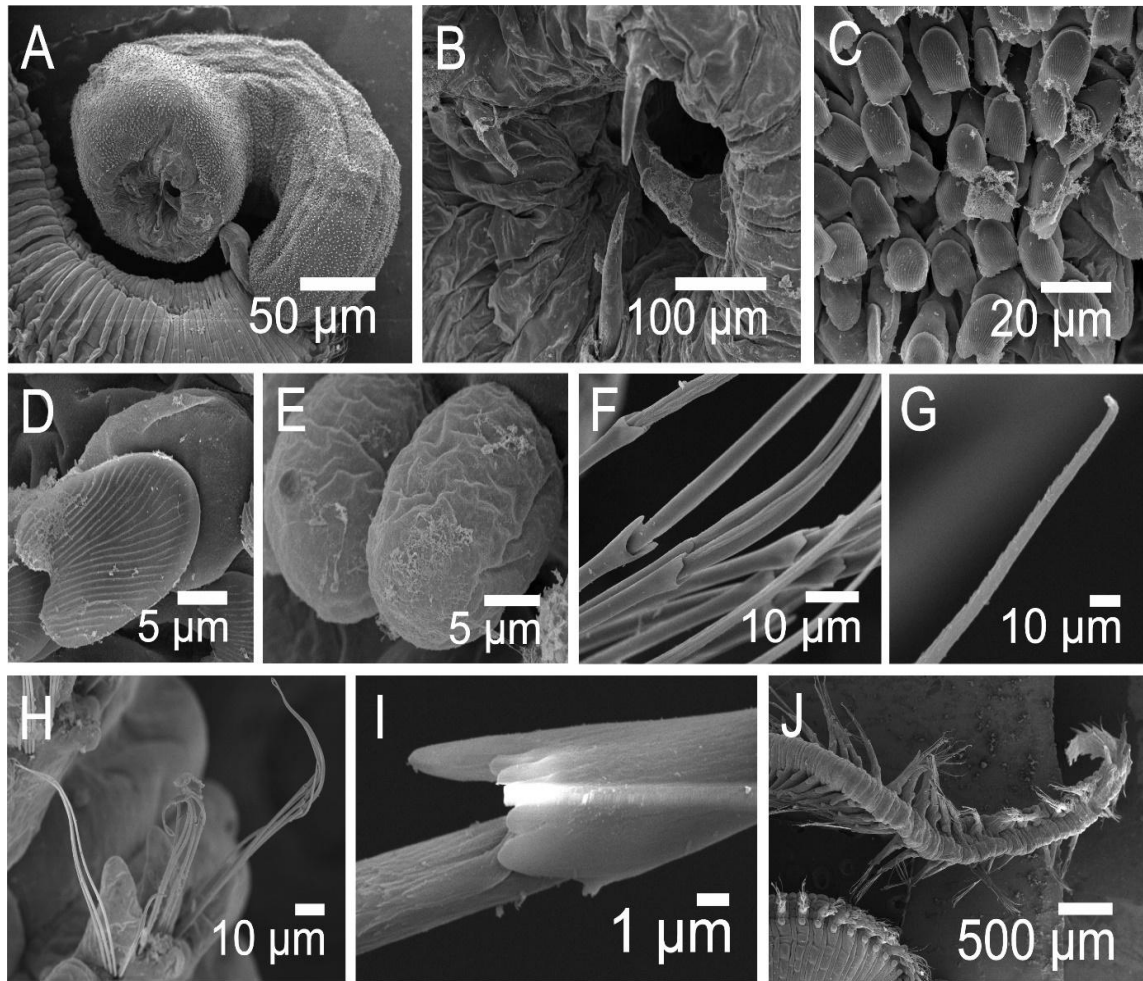


Figure 4.18: *Glycera alba*. A, proboscis, dorsal view; B, jaws, 4 hooked and chitinated black jaws; C & D, proboscideal papillae, leaf-shaped ridges on upper surface from top to bottom; E, proboscideal papillae, shorter and broader, rounded papillae; F, compound spiniger; G, compound spiniger, distal end; H, 1st parapodia; I, compound spiniger, middle margin; J, anterior end of body.

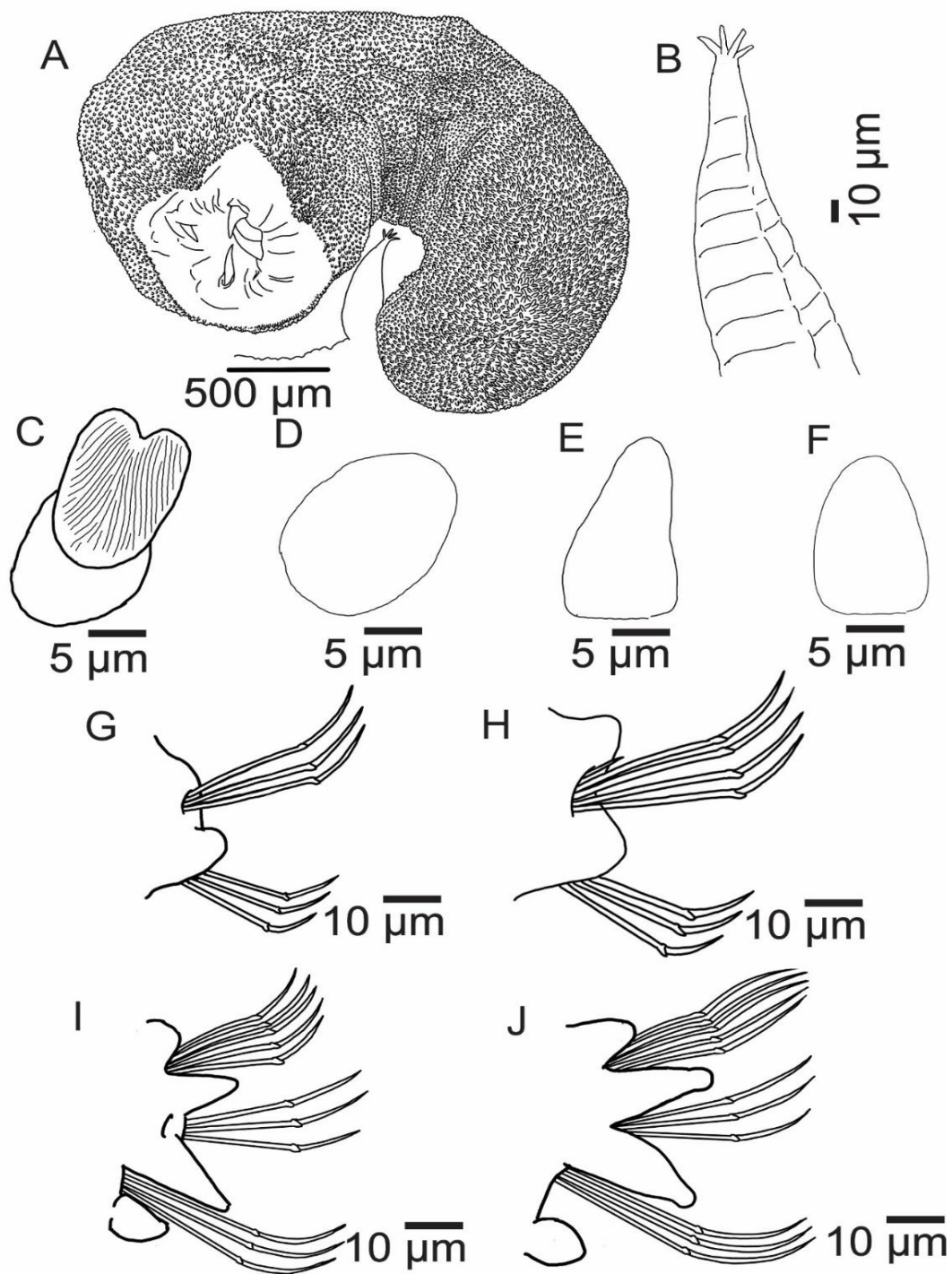


Figure 4.19: *Glycera alba*. A – C, proboscis with proboscideal papillae; B, prostomium with conical ring, bearing 4 small antennae at distal end; C – F; 3 types of proboscideal papillae; C, conical leaf-shaped ridges on posterior surface; D, rounded papillae with smooth surface; E & F; long, conical papillae with smooth surface; small and conical papillae with smooth surface; G, 1st parapodia; H, 2nd parapodia; I, 3rd parapodia; J, 4th parapodia.

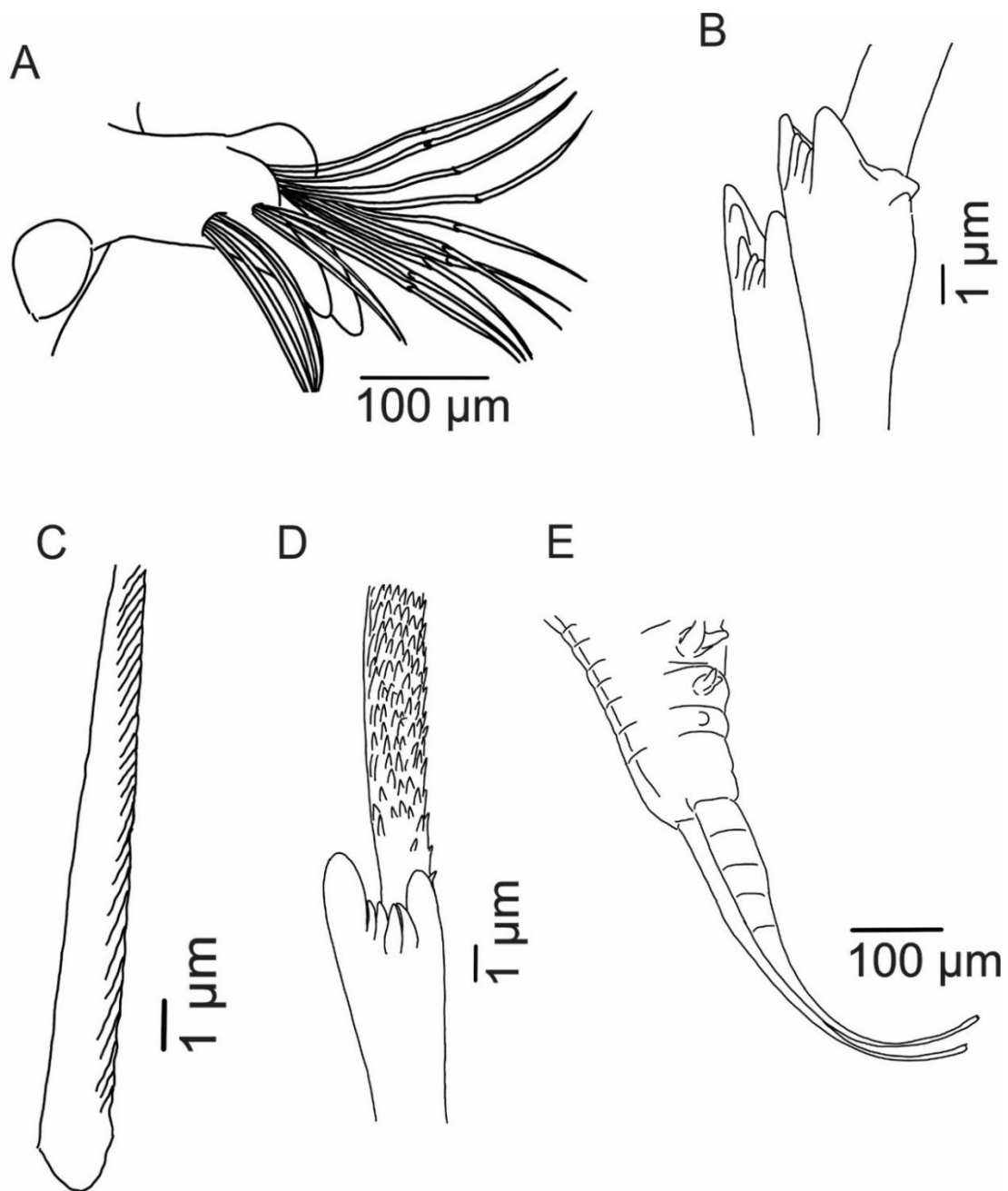


Figure 4.20: *Glycera alba*. A, middle parapodia; B, lateral view of compound spiniger, median part with distally stout finger-shaped opening on upper side of the spiniger and serrated capillary; C, compound spiniger, distal end; D, compound spiniger, dorsal view; E, distal end of the body, pygidium.

***Glycera tessellata* (Grube, 1863)**

(Figure 4.21 – 4.23)

Family Glyceridae, Grube, 1850

Genus *Glycera* Lamarck, 1818

Material Examined: Paratype (2 samples): Malaysia: East Malaysia: Sarawak: Bornean water: Buntal beach, Kuching: 1°42'40' N, 110°22'35" E, 20 March 2021; 2 paratypes (catalogue no.: ASD 04268 & ASD 04269). 1 km off Buntal coast. Out of 11 specimens collected in Buntal coast, only 2 complete specimens were chosen as paratypes for detailed morphological description.

Description: The whole body reaches up to 20 mm long, with up to 55 segments, has a width of 2 mm in the middle part of the body; preserved specimens in alcohol are pale yellowish. The body segments are biannulate and the anterior annulus shorter than the posterior annulus. The prostomium is conical, pointed and distinctly separated into about 20 rings; the terminal rings have four antennae. The proboscis is shorter and thicker in general compared to the posterior end of the body. The proboscis is about 3 mm in length and width of the anterior end of proboscis is about 1.5 mm. The proboscis is bell-shaped and densely covered with papillae. Proboscical papillae mainly digitiform with straight, median, longitudinal ridges; ailerons with deeply incised; parapodia of mid-body with slightly longer neuropodial than notopodial prechaetal lobes and two short postchaetal lobes; branchiae present. Mid body segments biannulate. Conical prostomium consisting of about 8 – 9 rings; terminal ring with four appendages and basal one with pair of nuchal organs. The proboscideal papillae consist of numerous, conical papillae with leaf-shaped ridges covering the posterior surface. The terminal of the proboscis is smooth with the presence of four hook-

shaped jaws with vertical incisions on its lateral side. The parapodia of the first two segments are uniramous, while the subsequent parapodia are biramous. Pointed-shaped dorsal cirrus from parapodium 2 eventually become digitiform-shaped continuing posteriorly. Knob-like dorsal cirri from the second parapodium are inserted most clearly at the base of the anterior parapodia, as well as on the body wall far above parapodial base in the mid-body, and again near the posterior base. Ventral cirri are well-developed in the middle part as it is bigger and rounder. Compound spinigers are thin and stouted, with distally crown-shaped opening on the spiniger tip. Presence of neurochaetae homogomph spinigers with two capillaries emerging from the middle, covered with spines or hairs. Only the dorsal cirri, prechaetal and postchaetal lobes, and ventral cirri present at final five segments. At the lateral side of the body end, the dorsal cirri, prechaetal and postchaetal lobes, and ventral cirri merges into one long digitiform. Two long, pointed and smooth tail-like papillae present at the pygidium of the body.

Distribution: Malaysia, New Zealand, Australia, Papua New Guinea, Vietnam, Phillipines, Andaman Sea, Mexico, Galapagos Island, San Francisco, Seattle, Gulf of Alaska; in this study, *Glycera tessellata* was found in 6 stations (T1S1, T1S2, T1S3, T2S1, T2S2, T2S3), which shows very fine sand to medium sand type of sediment.

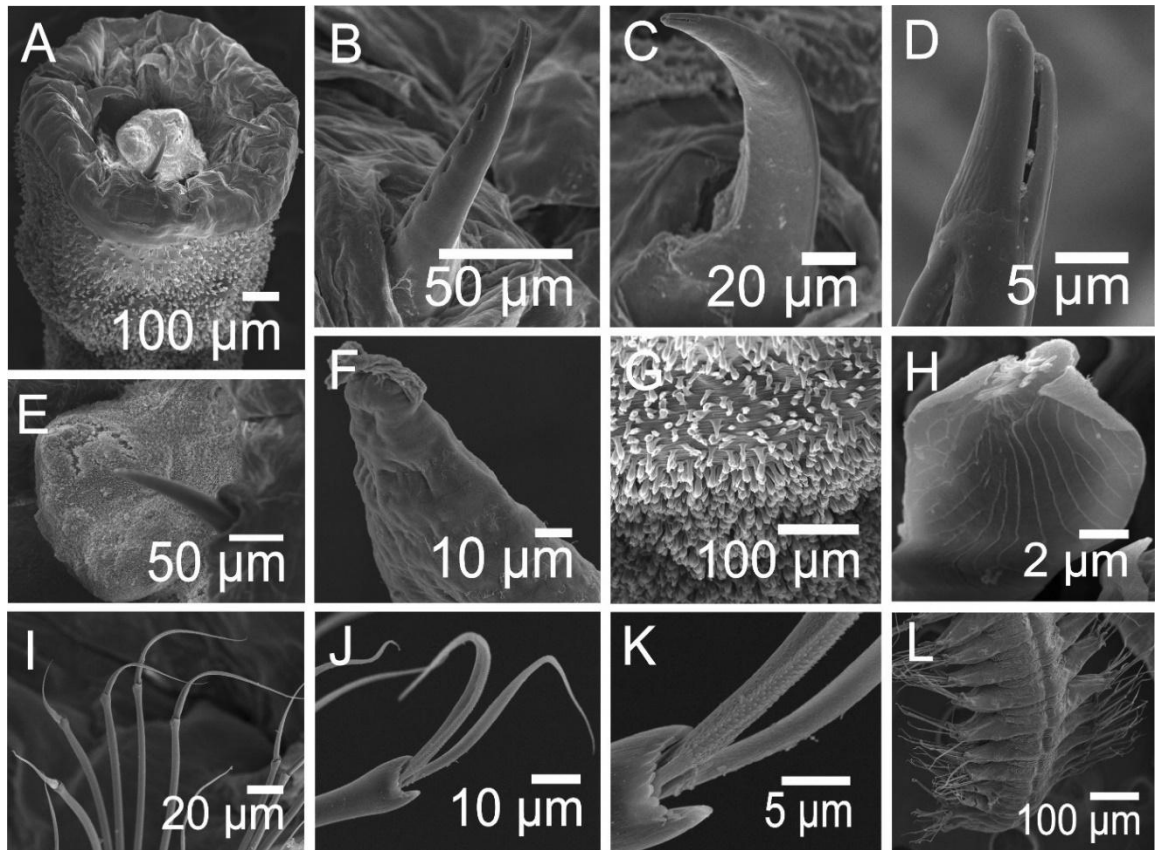


Figure 4.21: *Glycera tessellata*. A, proboscis, distal end; B – E, jaws, 4 hooked and chitinated black jaws; F, prostomium, distally branched to 4 antennae; G, proboscideal papillae; H, leaf-shaped ridges on posterior surface from top to bottom; I, compound spiniger; J, compound spiniger, two serrated capillaries taper to a fine tip; K, compound spiniger, middle margin; L, anterior end of body.

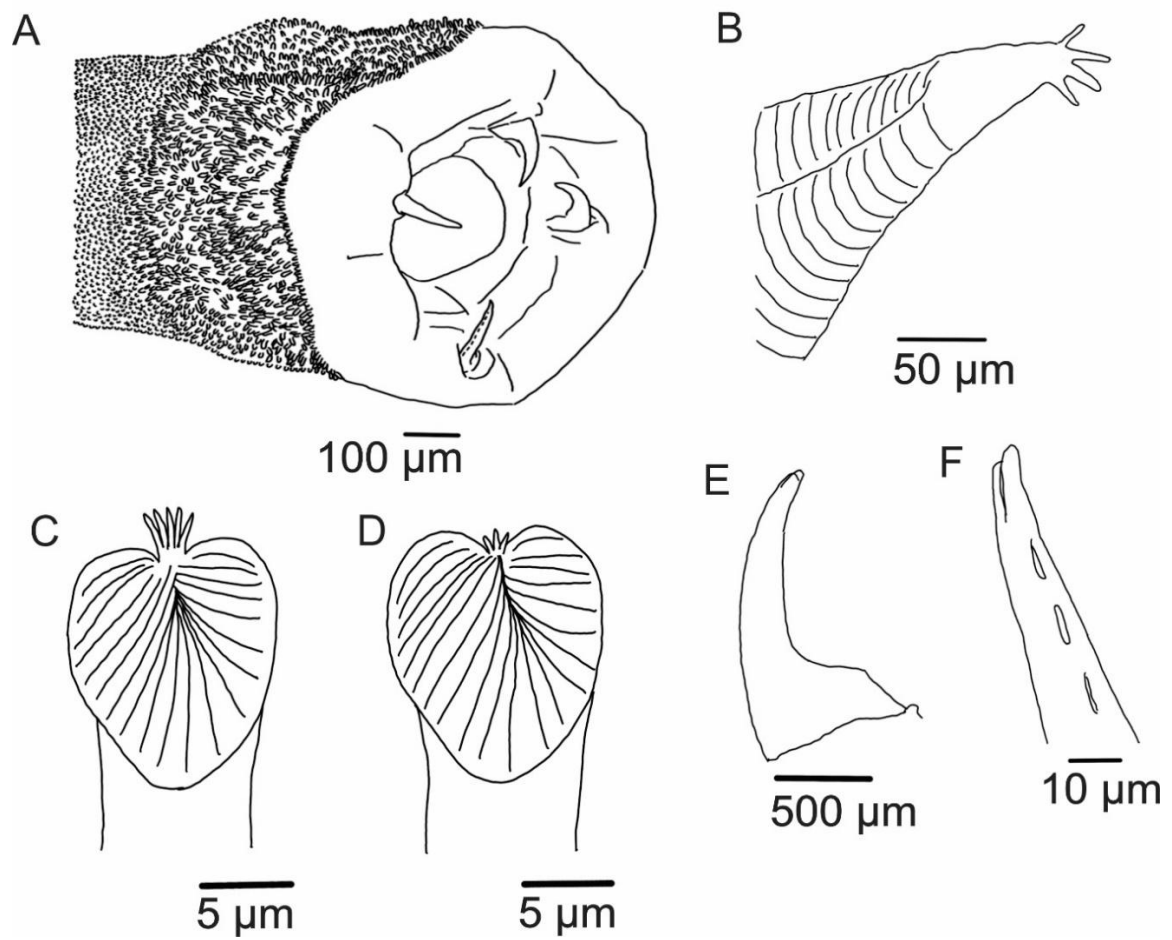


Figure 4.22: *Glycera tessellata*. A, proboscis; B, prostomium with conical rings and 4 small antennae; C & D, proboscideal papillae with leaf-shaped ridges on upper surface; E, lateral view of jaw; F, jaw with dorsal and lateral incision.

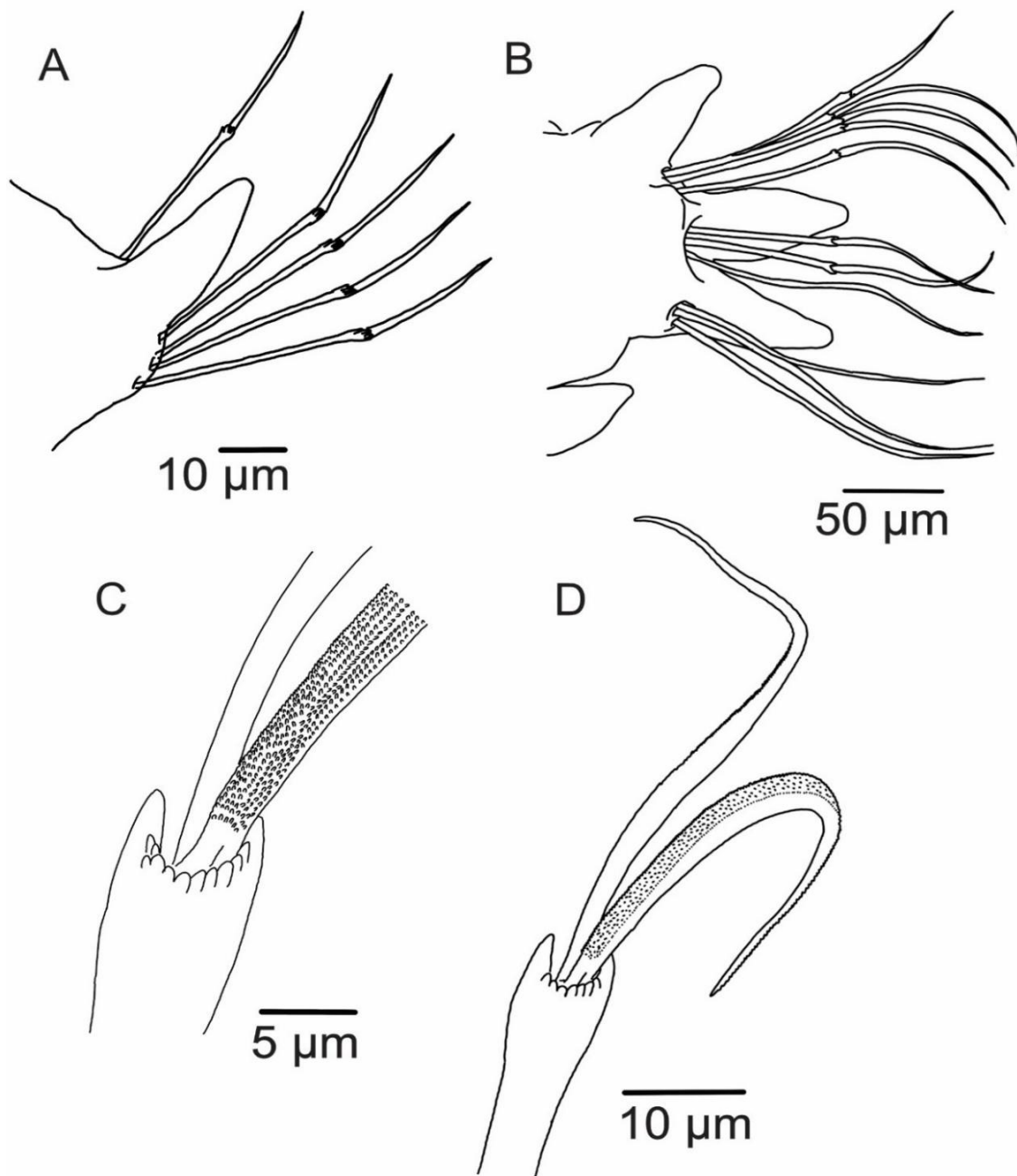


Figure 4.23: *Glycera tessellata*. A, 1st parapodia; B, 4th parapodia; C, compound spiniger, median part with distally crown-shaped on spiniger open end and serrated capillary.; D, compound spiniger, whole.

4.5.4.3 Family Nephtyidae Grube, 1850

Key to the Species of *Micronephthys* of Buntal Beach

Prostomium pentagonal with a straight anterior margin and rather long, slender and stoutened antennae. First chaetiger with small papilla-like dorsal cirrus and elongated ventral cirrus, following chaetigers with subspherical globular dorsal cirri and ovate ventral cirri. Setae include an anterior fan of laddered capillaries and a posterior fan of very long capillaries with spinulose margin and two to three short forked setae with spinules on their inner margin.....
.....*Micronephthys sphaerocirrata* (Wesenberg-Lund, 1949).

***Micronephthys sphaerocirrata* (Wesenberg-Lund, 1949)**

(Figure 4.24)

Family Nephtyidae Grube, 1850

Genus *Micronephthys* Friedrich, 1939

Material Examined: Paratype (2 samples): Malaysia: East Malaysia: Sarawak: Bornean water: Buntal beach, Kuching: 1°42'40' N, 110°22'35" E, 20 March 2021; 2 paratypes (catalogue no.: ASD 04270 & ASD 04271). 1 km off Buntal coast. Out of 7 specimens collected in Buntal coast, only 2 complete specimens were chosen as paratypes for detailed morphological description.

Description: The sample is about 10 mm in length and 1 mm in wide. Body is flattened and slightly square-shaped. Antennae and palps present. Prostomium pentagonal with a straight anterior margin and rather long, slender and stoutened antennae. Antennae and

palps short, tapering with knobbed tips, similar in length. Parapodia with vestigial lamellae, the first having a knobbed ventral cirrus. First chaetiger with small papilla-like dorsal cirrus and elongated ventral cirrus, similar in shape and length to palps, following chaetigers with subspherical globular dorsal cirri and ovate ventral cirri. Subsequent feet have small papilliform notopodial cirri but lack branchiae entirely. The setigerous lobe of the notopodium is bluntly conical without visible lamellae. The setigerous lobe of the neuropodium is conical with a small postsetal lamella. Setae include an anterior fan of laddered capillaries and a posterior fan of very long capillaries with spinulose margin and two to three short forked setae with spinules on their inner margin.

Distribution: China, Hong Kong, Indonesia, Singapore, Vietnam, Riau Island, Andaman Sea, Bay of Bengal, Sri Lanka, Mumbai, Persian Gulf, Cape Town, Senegal, Mediterranean Sea, Barcelona, Italy, Japan, South Korea, North Korea; in this study, *Micronephthys sphaerocirrata* was found in 3 stations (T1S3, T2S2, T2S3), which shows very fine sand to medium sand type of sediment.

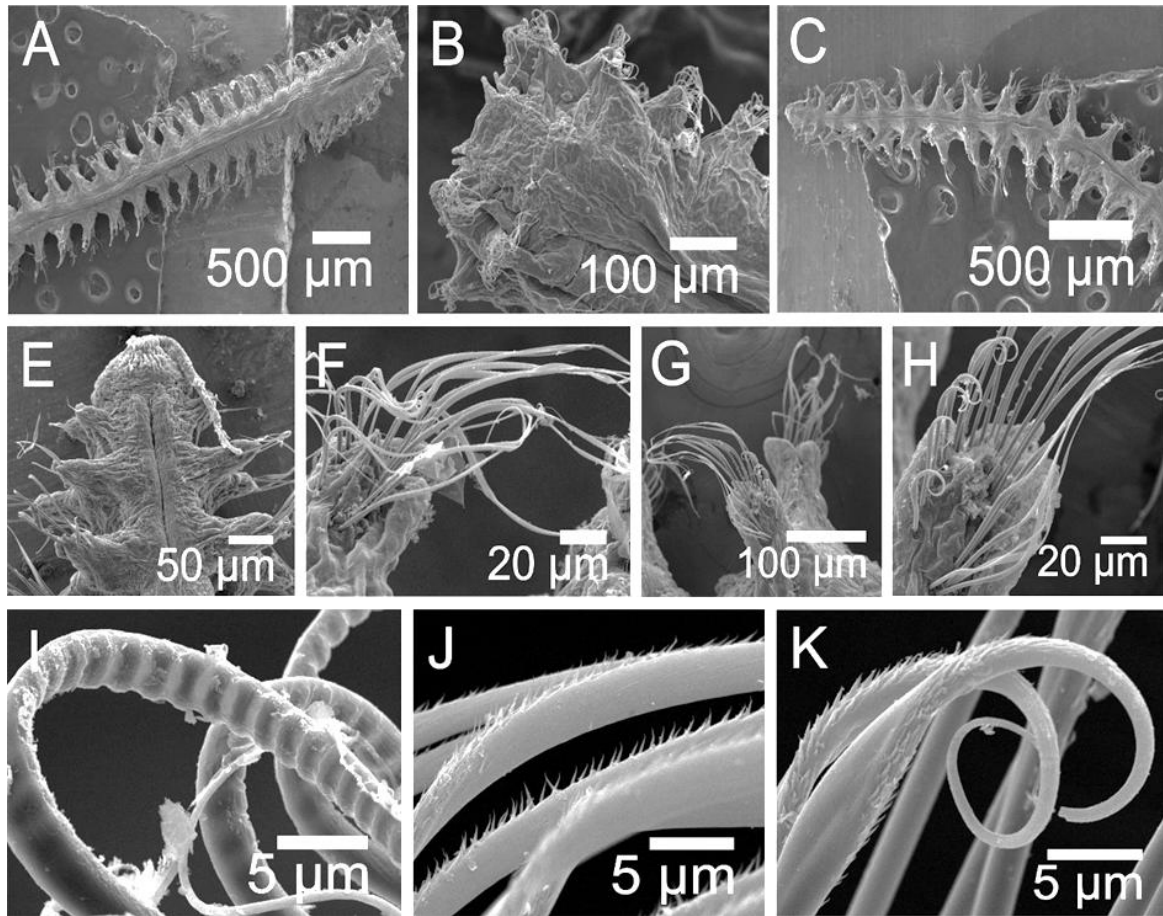


Figure 4.24: *Micronephthys sphaerocirrata*. A, whole body; B, head; C, anterior end of body; D, pygidium; E, 1st parapodia; F, 3rd parapodia; G, 10th notopodium; H, subacicular setae on 1st parapodia, laddered; I, post – acicular capillary setae on 10th parapodia; J, serrated chaetae with thick base and taper to a thin end.

4.5.4.4 Family Onuphidae Kinberg, 1865

Key to the Species of *Onuphis* of Buntal Beach

1. Tube dwelling with long and slender body. Prostomium subtriangular with pair of conical frontal lips. Presence of branchiae from first chaetiger. Presence of ceratophores and palps at anterior end. Presence of pseudocompound hooks, winged capillaries, comb-setae and acicular seta.....2
2. A) Dark-coloured body. Presence of tridentate hooded pseudocompound appendaged hooks extending over chaetiger 3 to 5 and three bidentate acicular setae on dorsal and ventral part along the side of the body 3
B) Pale body. Presence of tridentate hooded pseudocompound appendaged hooks extending over chaetiger 1 to 4 and two bidentate acicular setae on dorsal and ventral part along the side of the body.....4
3. Brown pigment of 4 – 6 circular spots on underside of ceratophores. Frontal palp thick and stoutened. Tentacular cirri inserted dorso-laterally. Branchiae increase to two filaments by the 22nd to 24th foot. Presence of three hooded bidentate acicular setae with short stalk.....*Onuphis eremita* (Audouin & Milne Edwards, 1833)
4. A) Several minute papillae at base of palpal of median antennae. Frontal palp conical. Tentacular cirri inserted dorsally at the upper side of peristomium. Branchiae increase to two filaments by the 20nd to 22th foot. Presence of two hooded bidentate acicular setae from short stalk to long stalk continuing posteriorly.....*Onuphis* sp. 1

B) Body is elongated and thin. Frontal palp conical and stouted. Tentacular cirri inserted dorsally at the upper side of peristomium. Branchiae increase to two filaments by the 15th to 20th foot. Presence of two hooded bidentate acicular setae with short stalk.....*Onuphis* sp. 2

***Onuphis eremita* (Audouin & Milne Edwards, 1833)**

(Figure 4.25 – Figure 4.28)

Family Onuphidae Kinberg, 1865

Genus *Onuphis* Audouin & Milne Edwards, 1833

Material Examined: Paratype (2 samples): Malaysia: East Malaysia: Sarawak: Bornean water: Buntal beach, Kuching: 1°42'40' N, 110°22'35" E, 20 March 2021; 2 paratypes (catalogue no.: ASD 04272 & ASD 04273). 1 km off Buntal coast. Out of 19 specimens collected in Buntal coast, only 2 complete specimens were chosen as paratypes for detailed morphological description.

Description: The sample is about 15 mm to 20 mm long, with about 80 segments. Tube is thin and encrusted with sand and shell particle debris. The body is iridescent, dark in colour, with brown or dark pattern continuing posteriorly. Brown pigment forming 4 – 6 circular spots on underside of ceratophores of antennae and palps. Prostomium subtriangular with pair of conical frontal lips. Prostomium anteriorly extended with anterior segment slightly rounded compared to the rest of the body. Presence of a pair of thick, triangular and stouted frontal palp. Median occipital antennae shorter than the inner lateral antennae but its ceratophore is long and closely ringed. Ceratophores are closely ringed, palpal ceratophores

with 15 – 20 rings, median antennae with 20 – 25 rings and lateral antennae with 25 – 30 rings. Tentacular cirri inserted dorso-laterally and shorter than the peristomial segment. Branchiae from the first foot as simple filaments; they increase to two filaments by the 22nd to 24th foot, reach a maximum of four to five filaments. Awl-shaped ventral cirri in first four to five chaetigers, distinct slender and pointed postchaetal lobes in first 10 – 15 chaetigers. Anterior hooded pseudocompound appendaged hooks have three teeth; they extend over chaetiger 1 to 5. Posterior setae include winged capillaries, comb-setae with flat, distally oblique blades with about 12 teeth and three bidentate acicular setae with short stalk, two acicular setae located dorsally and one aciculae seta on ventral side. Subacicular hooks start from chaetiger 15. Presence of 4 anal cirri on pygidium, 2 cirri shorter and 2 cirri longer in length.

Distribution: Indonesia, India, Korea, Brazil, USA, France, in this study, *Onuphis eremita* was found in 4 stations (T1S2, T1S3, T2S2, T2S3), which shows very fine sand to medium sand type of sediment.

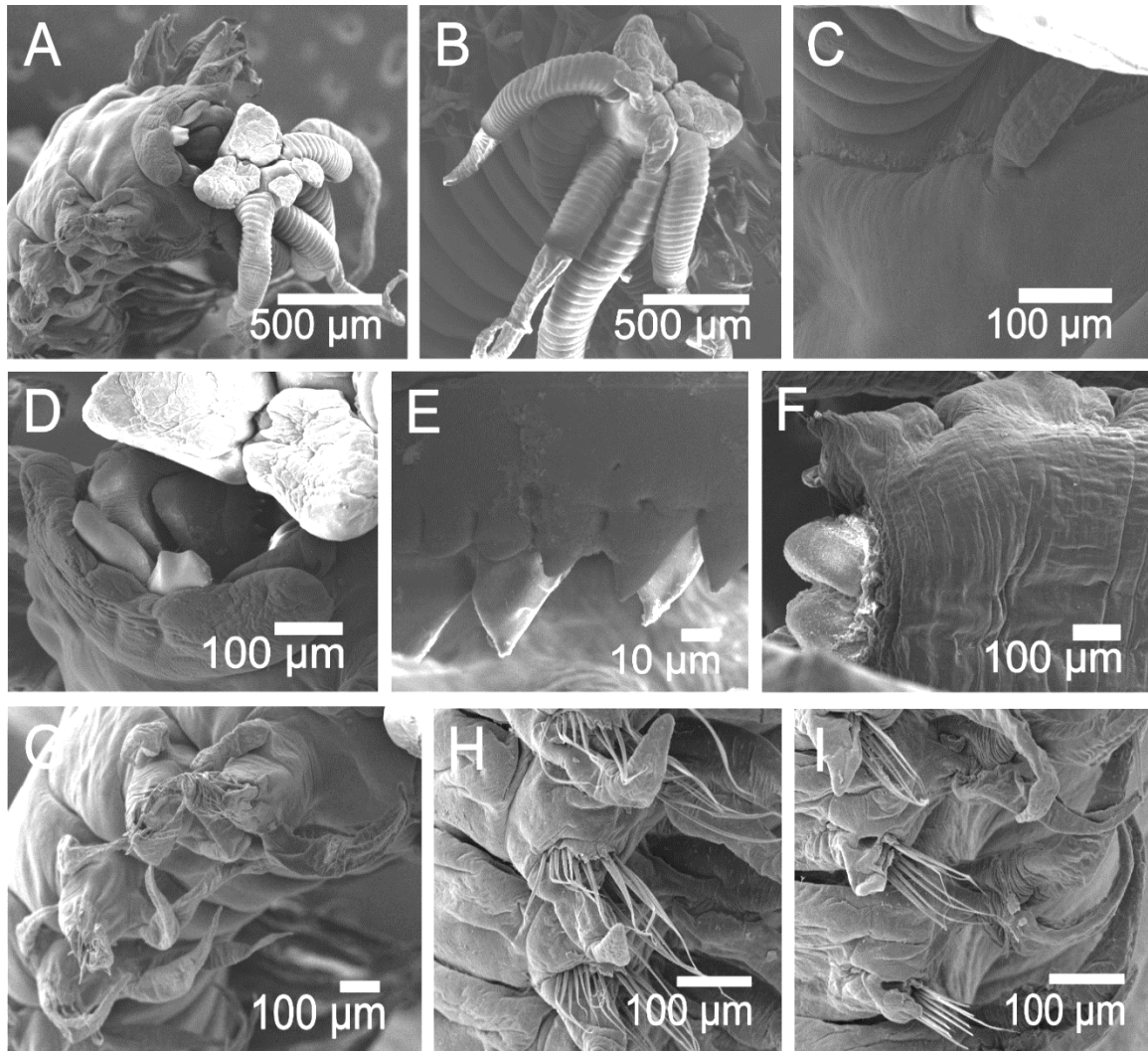


Figure 4.25: *Onuphis eremita*. A, head; B, antennae, dorsal view; C, tentacular cirri; D, upper and lower lips; E, maxillae; F, pygidium; G, 1st to 4th parapodia; H, 7th to 9th parapodia; I, 14th to 15th parapodia.

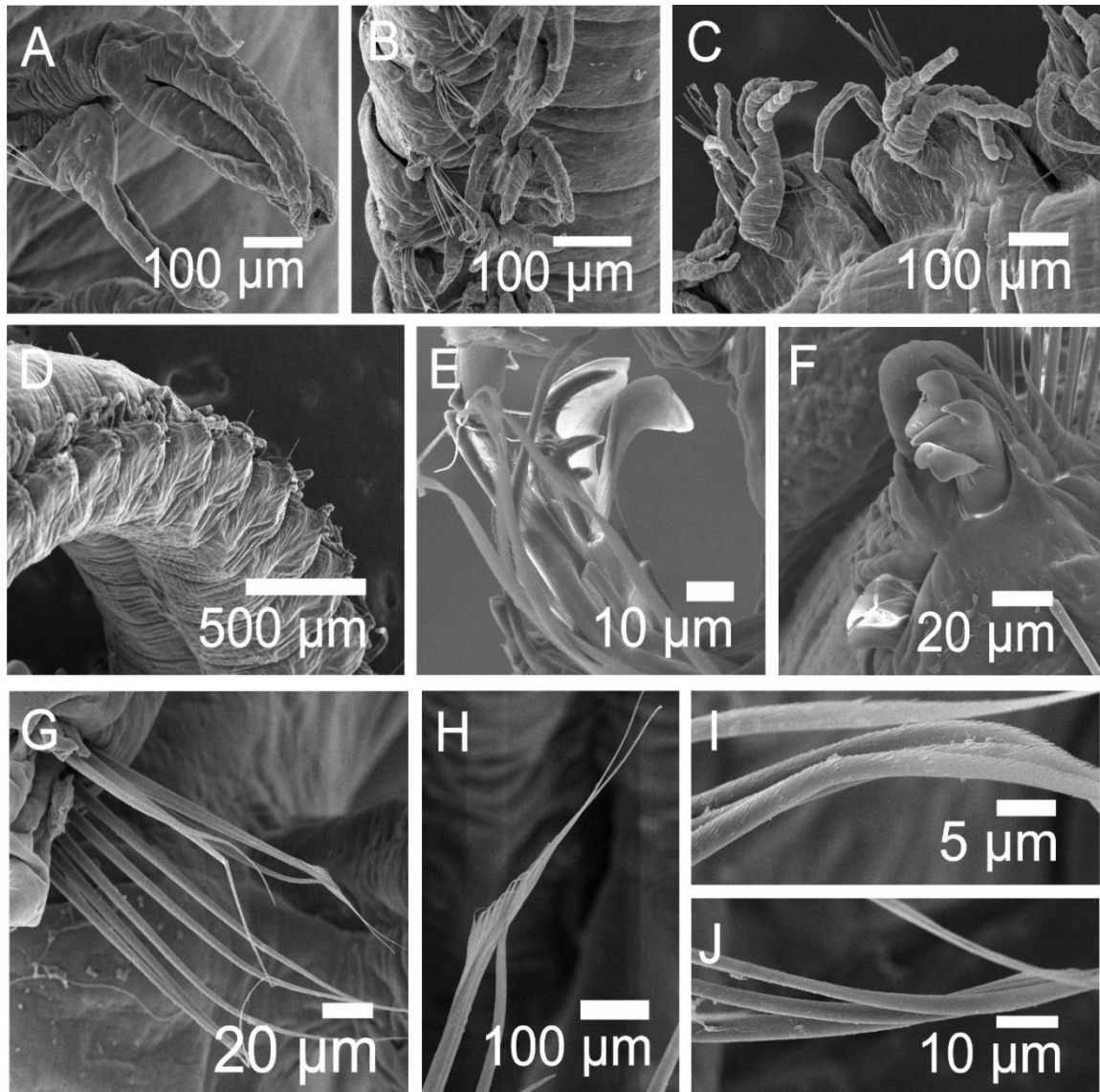


Figure 4.26: *Onuphis eremita*. A, branchiae of 15th parapodia; B, branchiae of 24th to 26th parapodia; C, branchiae of 40th to 41st parapodia; D, anterior end of the body; E, tridentate pseudocompound hooked chaetae on 1st parapodia; F, bidentate acicular setae; G, neurochaetae on 40th parapodia; H, pectinate chaetae and capillaries; I & J, capillaries slightly serrated on one side on upper margin.

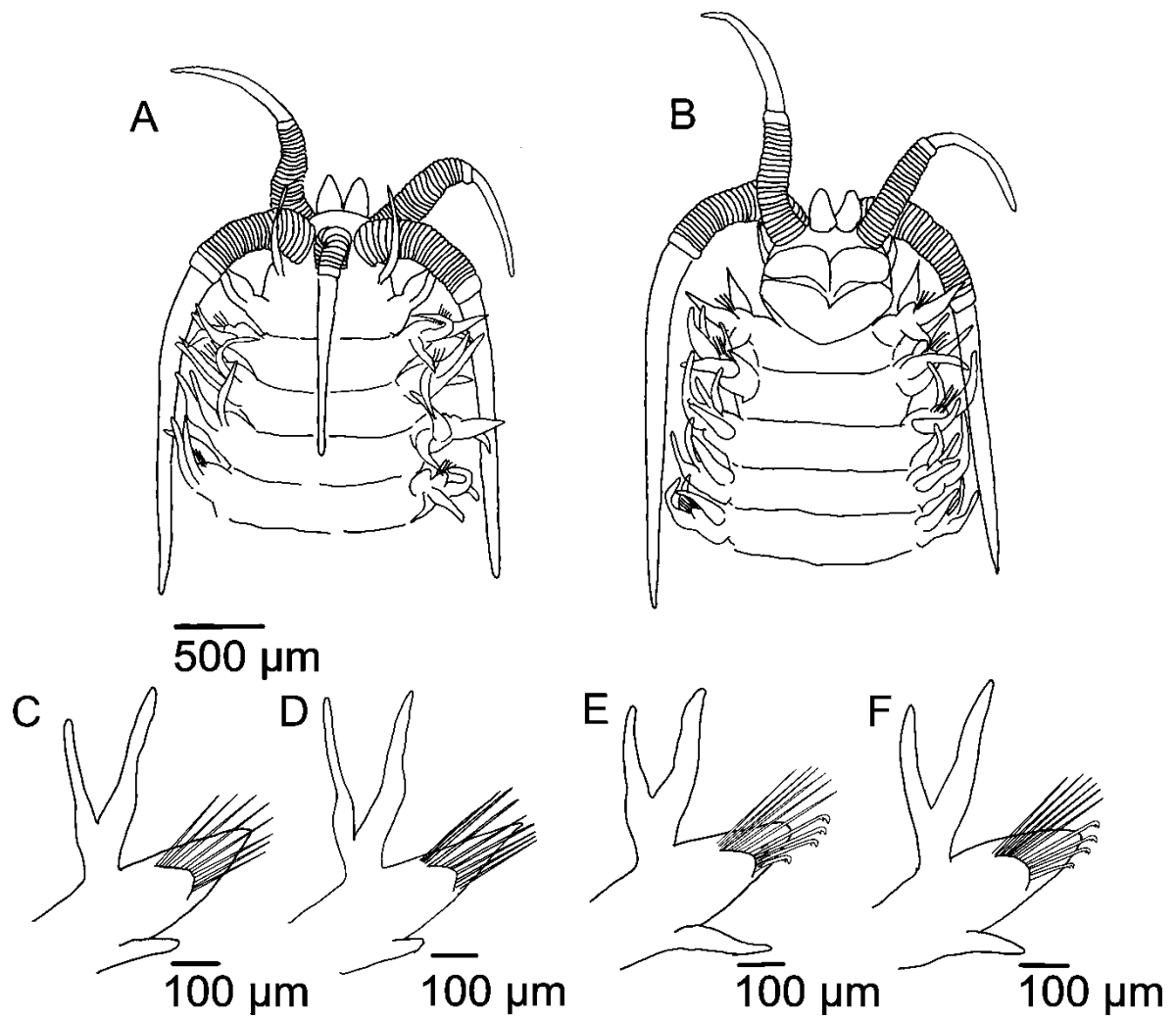


Figure 4.27: *Onuphis eremita*. A & B, dorsal and ventral view of the posterior body part; C – F, 1st to 4th parapodia.

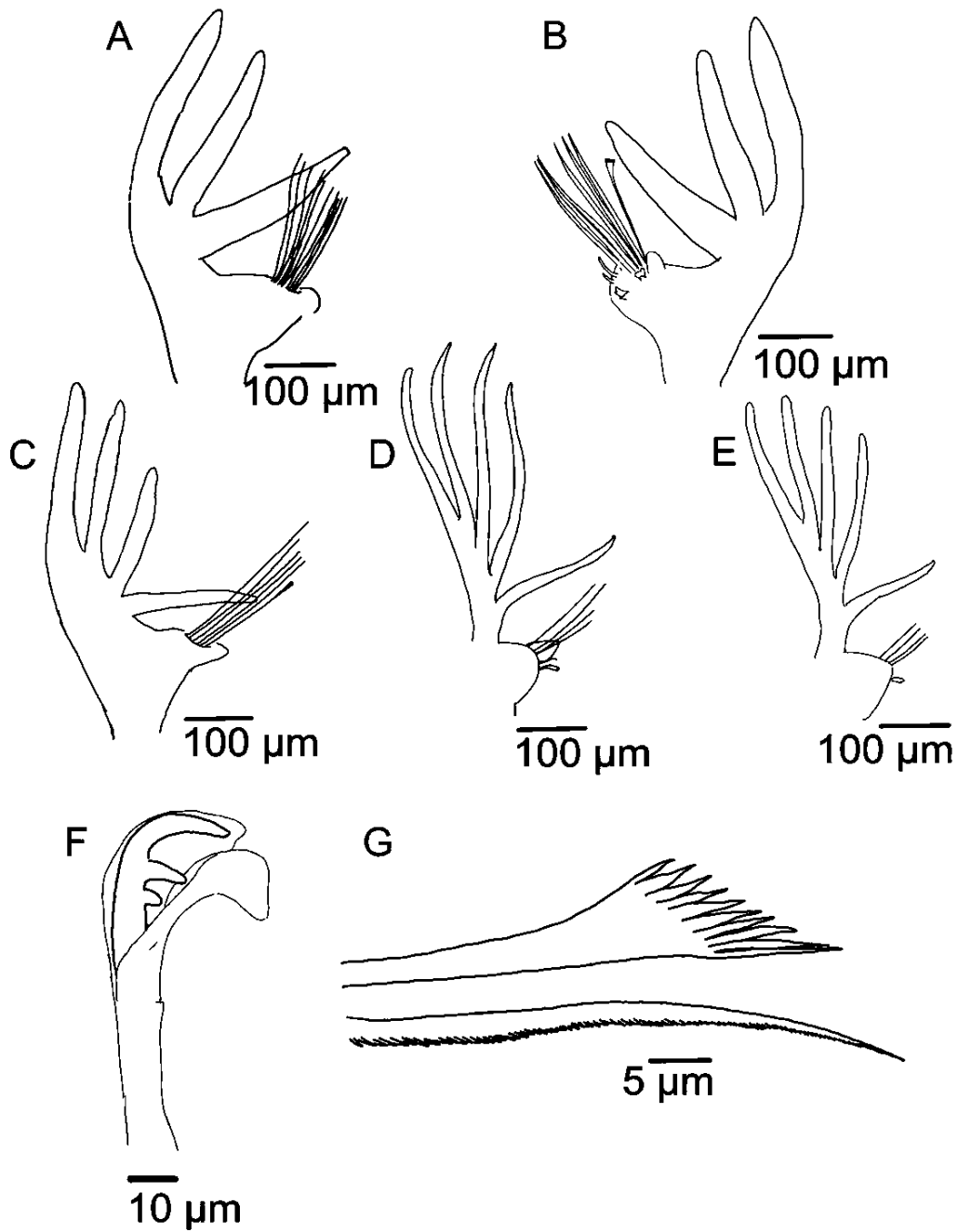


Figure 4.28: *Onuphis eremita*. A & B, 45th parapodia, left and right view; C, 50th parapodia; D, 55th parapodia; E, 70th parapodia; F, tridentate pseudocompound hooked chaetae on 1st parapodia; G, distal end of pectinate chaetae and serrated capillary.

***Onuphis* sp. 1**

(Figure 4.29 – Figure 4.34)

Family Onuphidae Kinberg, 1865

Genus *Onuphis* Audouin & Milne Edwards, 1833

Material Examined: Paratype (2 samples): Malaysia: East Malaysia: Sarawak: Bornean water: Buntal beach, Kuching: 1°42'40' N, 110°22'35" E, 20 March 2021; 2 paratypes (catalogue no.: ASD 04274 & ASD 04275). 1 km off Buntal coast. Out of 7 specimens collected in Buntal coast, only 2 complete specimens were chosen as paratypes for detailed morphological description.

Description: The sample is about 8 mm to 10 mm long, with about 55 segments. Tube is thin with sand debris. Body is pale in colour, white or pale yellow after alcohol immersion. Prostomium subtriangular with a pair of conical frontal lips. Prostomium anteriorly extended with anterior segment slightly elongated compared to the rest of the body. Presence of a pair of conical frontal palp. Presence of subulate minute papillae at base of palpal of median antennae. Lateral antennae are two times the length of median occipital antennae. Ceratophores are strongly ringed with deep circular ridges, palpal ceratophores with 15 – 20 rings, median antennae with 10 – 12 rings and lateral antennae with 25 – 30 rings. Tentacular cirri inserted dorsally at the upper side of peristomium and longer than the peristomial segment. Awl-shaped ventral cirri in first four to five chaetigers, distinct slender and pointed post-chaetal lobes in first 10 -15 chaetigers. Branchiae from the first foot as simple filaments; they increase to two filaments by the 20nd to 22th foot, reach a maximum of four to five filaments. Anterior hooded pseudocompound appendaged hooks have three teeth; they extend over chaetiger 1 to 4. Posterior setae include winged capillaries, comb-

setae with flat, distally oblique blades with about 10 teeth and two hooded bidentate acicular setae. Subacicular hooks start from chaetiger 15, from short hooded stalk to hooded with long stalk continuing posteriorly.

Distribution: Global distribution is unknown, in this study, *Onuphis* sp. 1 was found in 2 stations (T1S2, T2S2), which shows very fine sand to medium sand type of sediment.

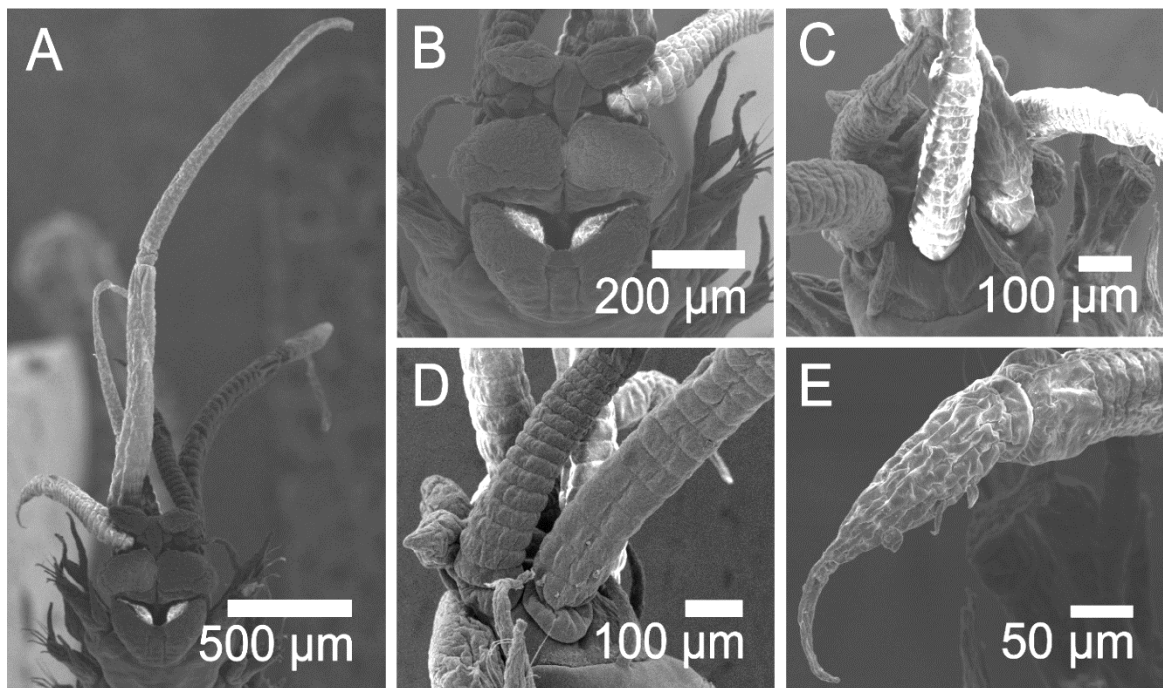


Figure 4.29: *Onuphis* sp. 1. A, head, dorsal view with a pair of frontal lips; B, prostomium; C, antennae and peristomial cirri; D, anterior end, lateral view; E, subulate minute papillae at the base of median antennae.

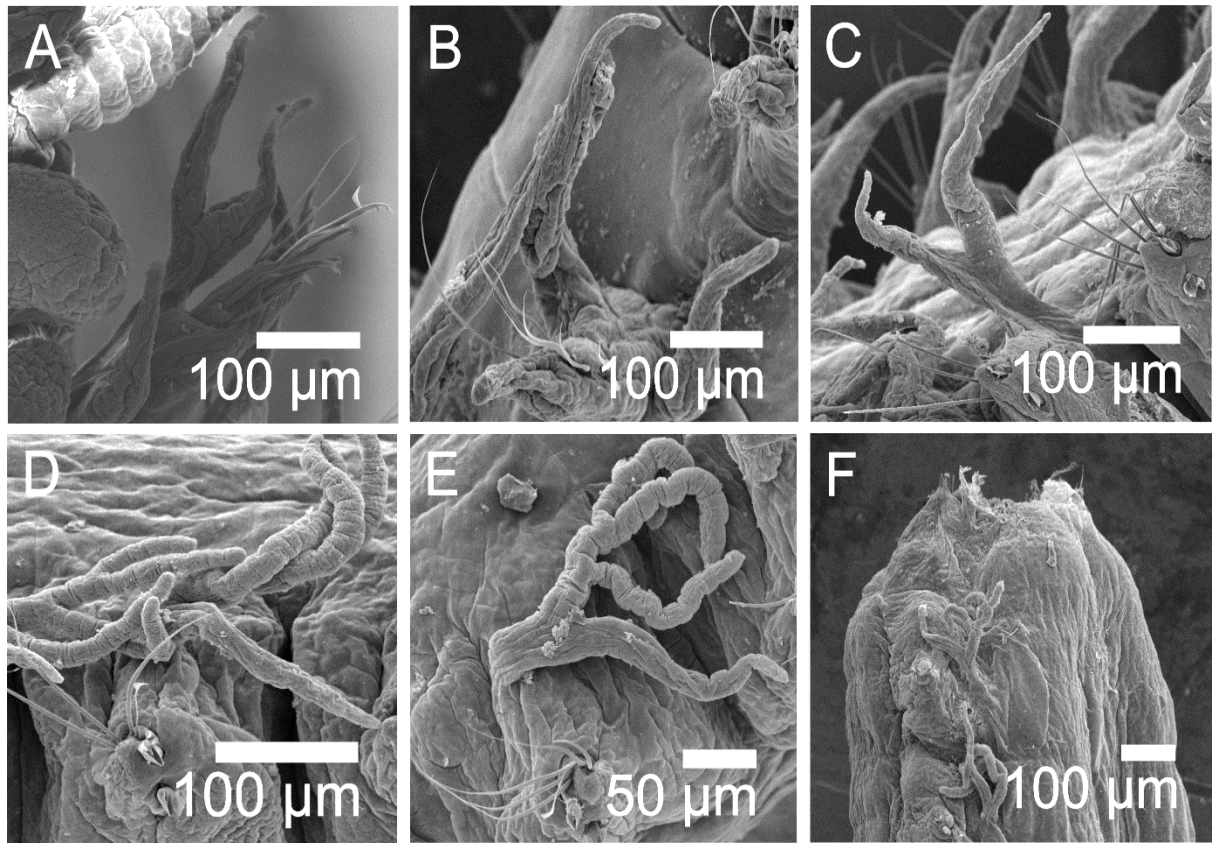


Figure 4.30: *Onuphis* sp. 1. A, 1st parapodia, dorsal view; B, 2nd parapodia, lateral view; C, dorsal cirri and single branchiae on 13th parapodia; D, branchiae on 25th segment; E, branchiae on 35th segment; F, pygidium.

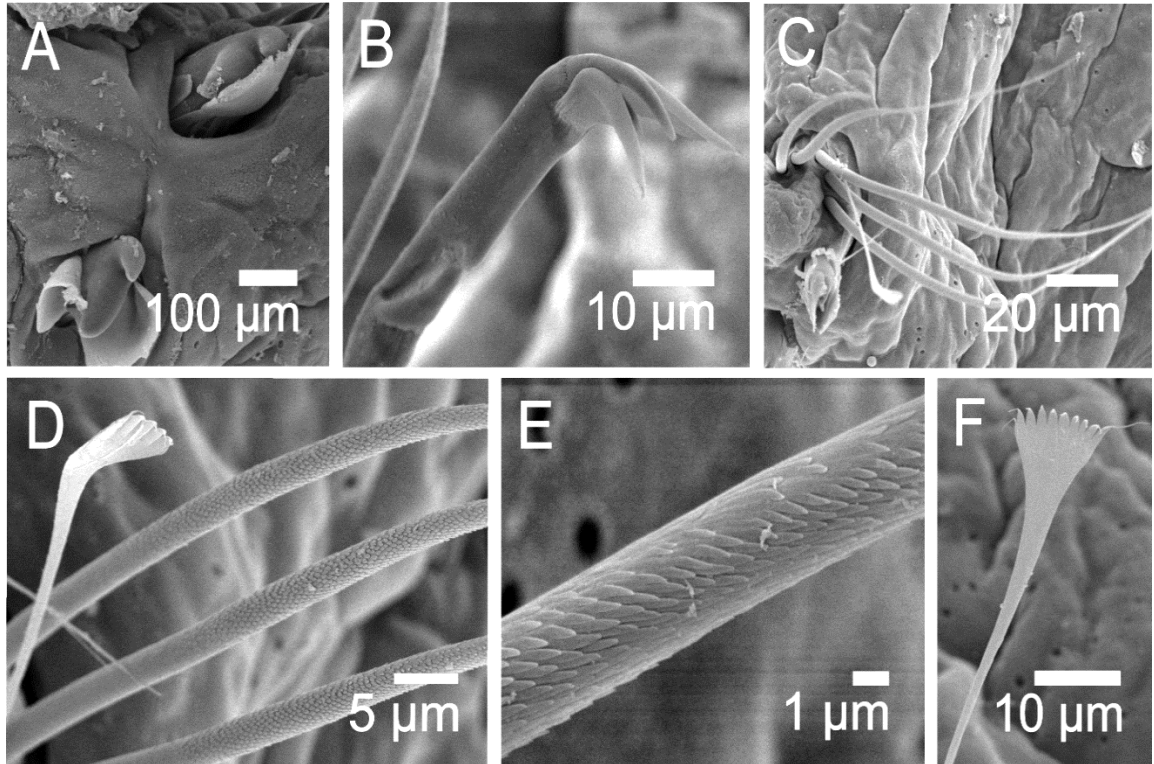


Figure 4.31: *Onuphis* sp. 1. A, bidentate acicular setae on 15th parapodia; B, tridentate pseudocompound hooked chaetae on 1st parapodia; C, neurochaetae on 35th segment; D, pectinate chaetae and denticulated capillaries on 15th parapodia; E, serrated capillaries on 15th parapodia; F, pectinate chaetae with 10 teeth from 15th parapodia.

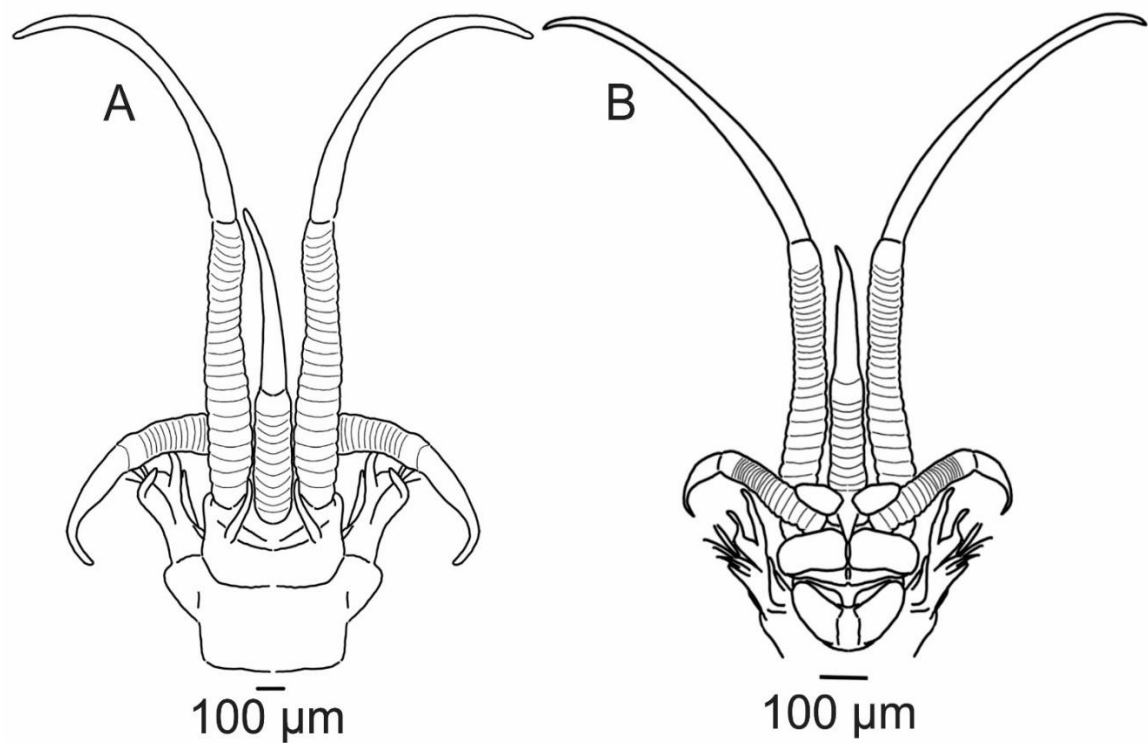


Figure 4.32: *Onuphis* sp. 1. A, dorsal view of head; B, ventral view of head.

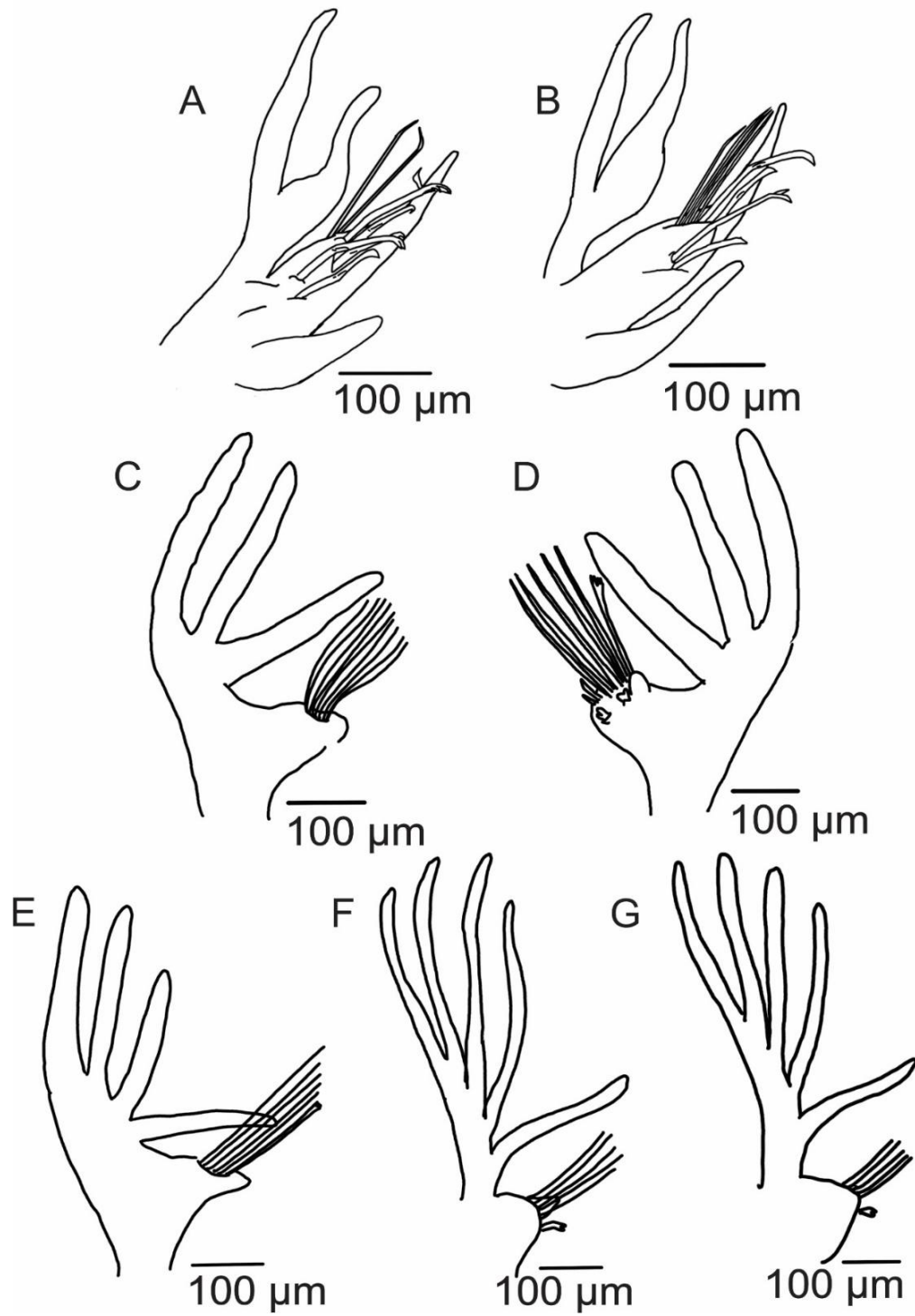


Figure 4.33: *Onuphis* sp. 1. A, 1st parapodia; B, 2nd parapodia; C, left view of 18th parapodia; D, right view of 18th parapodia; E, Branchiae of 23rd parapodia; F, Branchiae of 30th parapodia; G, Branchiae of 55th parapodia

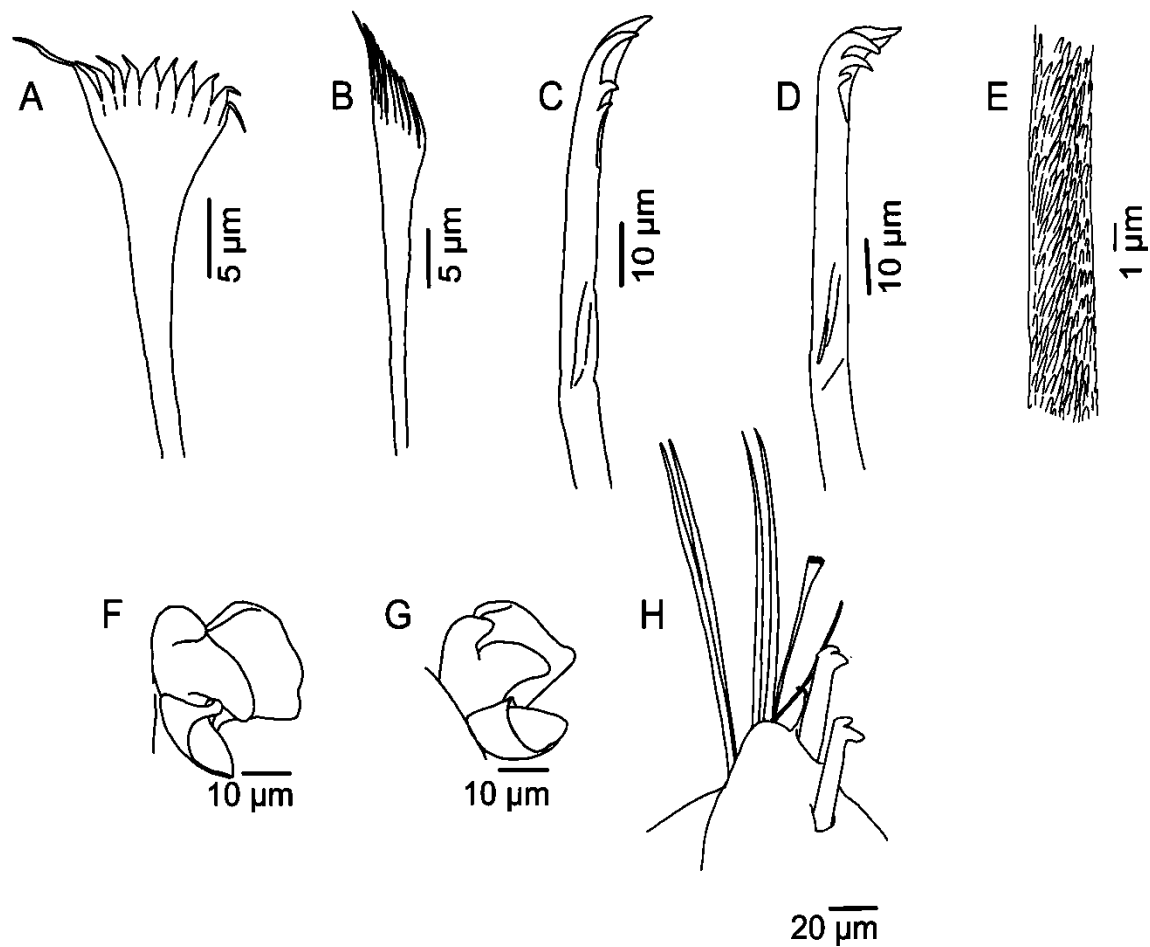


Figure 4.34: *Onuphis* sp. 1. A & B, pectinate of 15th parapodia and 20th parapodia; C & D, tridentate pseudocompound chaetae on 1st parapodia and 4th parapodia; E, serrated capillary on 15th parapodia; F & G, bidentate subacicular chaetae on with short stalk from 15th parapodia; H, bidentate subacicular chaetae with long stalk, pectinate chaetae and long, thin capillaries on 45th parapodia.

***Onuphis* sp. 2**

(Figure 4.35 – Figure 4.37)

Family Onuphidae Kinberg, 1865

Genus *Onuphis* Audouin & Milne Edwards, 1833

Material Examined: Paratype (2 samples): Malaysia: East Malaysia: Sarawak: Bornean water: Buntal beach, Kuching: 1°42'40' N, 110°22'35" E, 20 March 2021; 2 paratypes (catalogue no.: ASD 04276 & ASD 04277). 1 km off Buntal coast. Out of 5 specimens collected in Buntal coast, only 2 complete specimens were chosen as paratypes for detailed morphological description.

Description: The sample is about 18 mm to 20 mm long, with about 85 segments. Tube is thin with sand and shell particle debris. The body is elongated, thin and pale in colour after alcohol immersion. Numerous circular pores of sensory buds forming on underside of ceratophores of antennae and palps. Prostomium subtriangular with a pair of conical frontal lips. Prostomium located anteriorly with slightly stouted compared to the rest of the body. Lateral antennae longer than the median occipital antennae. Palpal ceratophores with 20 – 23 rings, median antennae with 15 – 20 rings and lateral antennae with 25 – 30 rings. Tentacular cirri inserted dorsally at the centre of peristomium and longer than the peristomial segment. Branchiae from the first foot as simple filaments; they increase to two filaments by the 15th to 20th foot. Anterior hooded pseudocompound appendaged hooks have three teeth; they extend over chaetiger 1 to 4. Posterior setae include winged capillaries, comb-setae with flat, distally oblique blades with about 8 – 10 teeth and two hooded bidentate acicular setae with short stalk. Subacicular hooks start from chaetiger 15. Presence of 4 anal cirri on pygidium, 2 cirri shorter and 2 cirri longer in length.

Distribution: Global distribution is unknown, in this study, *Onuphis* sp. 2 was found in 3 stations (T1S2, T2S2 & T2S3), which shows very fine sand to medium sand type of sediment.

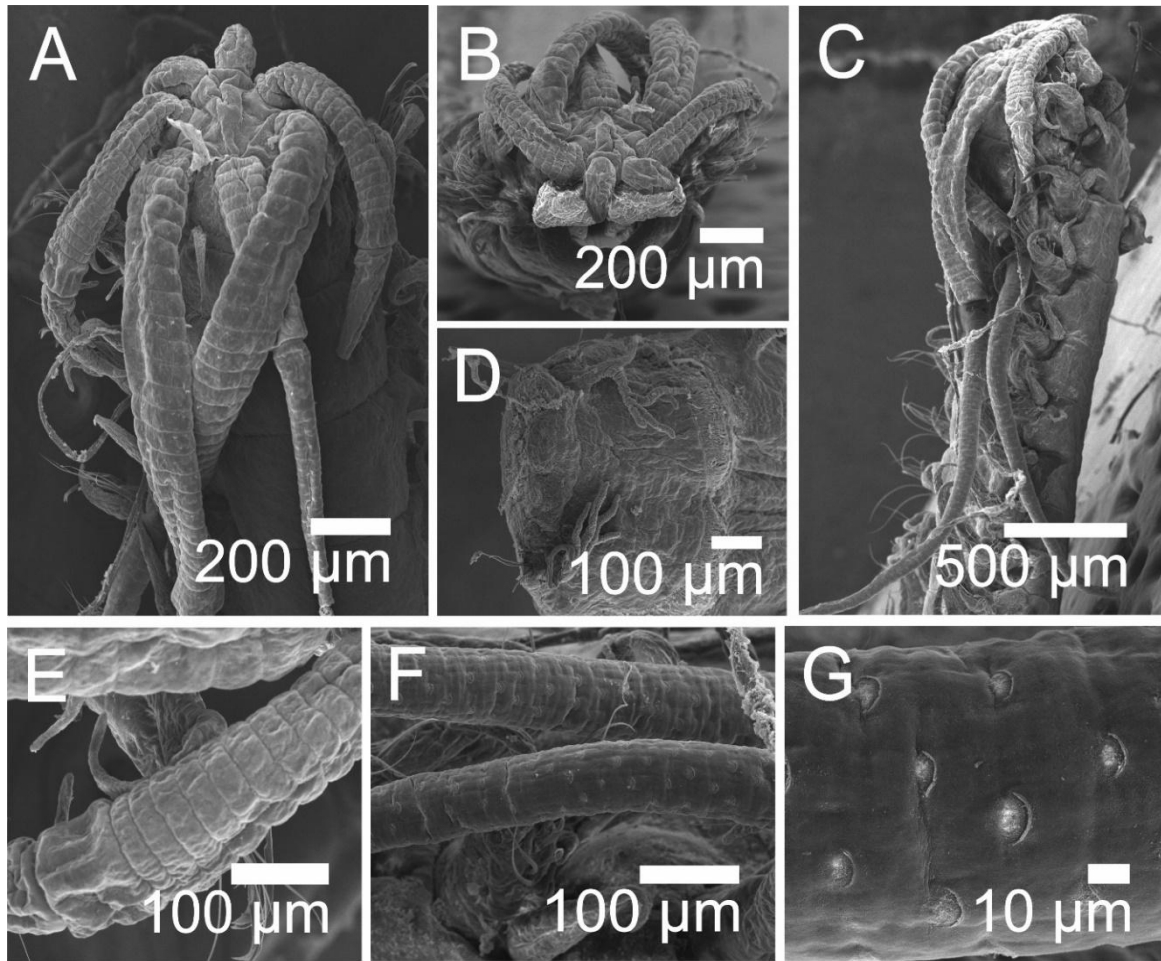


Figure 4.35: *Onuphis* sp. 2. A, head with antennae; B, anterior view of the head; C, lateral view of the body; E, ceratophore rings on antennae; F, under side of antennae and palp with numerous semi – circular pores of sensory bud; G, semi – circular pore of sensory bud.

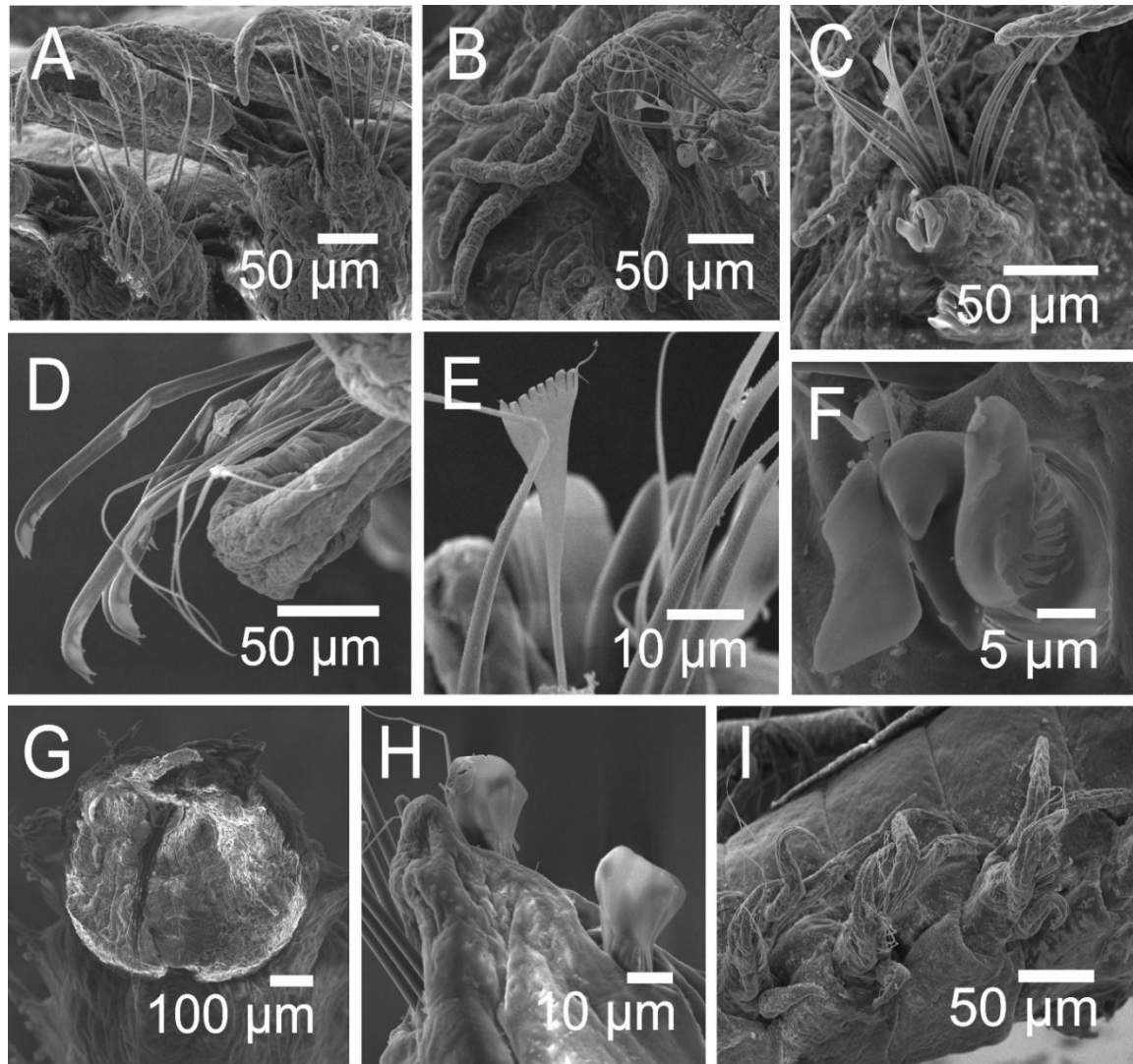


Figure 4.36: *Onuphis* sp. 2. A, 20th and 21st parapodia; B, branchiae of 45th parapodia; C, chaetae of 45th parapodia; D, tridentate pseudocompound chaetae of 1st parapodia; E, pectinate chaetae of 15th parapodia; F, bidentate subacicular chaetae of 15th parapodia; G, posterior end, pygidium; H, bidentate acicular chaeta on 45th parapodia; I, lateral view of 15th to 17th parapodia.

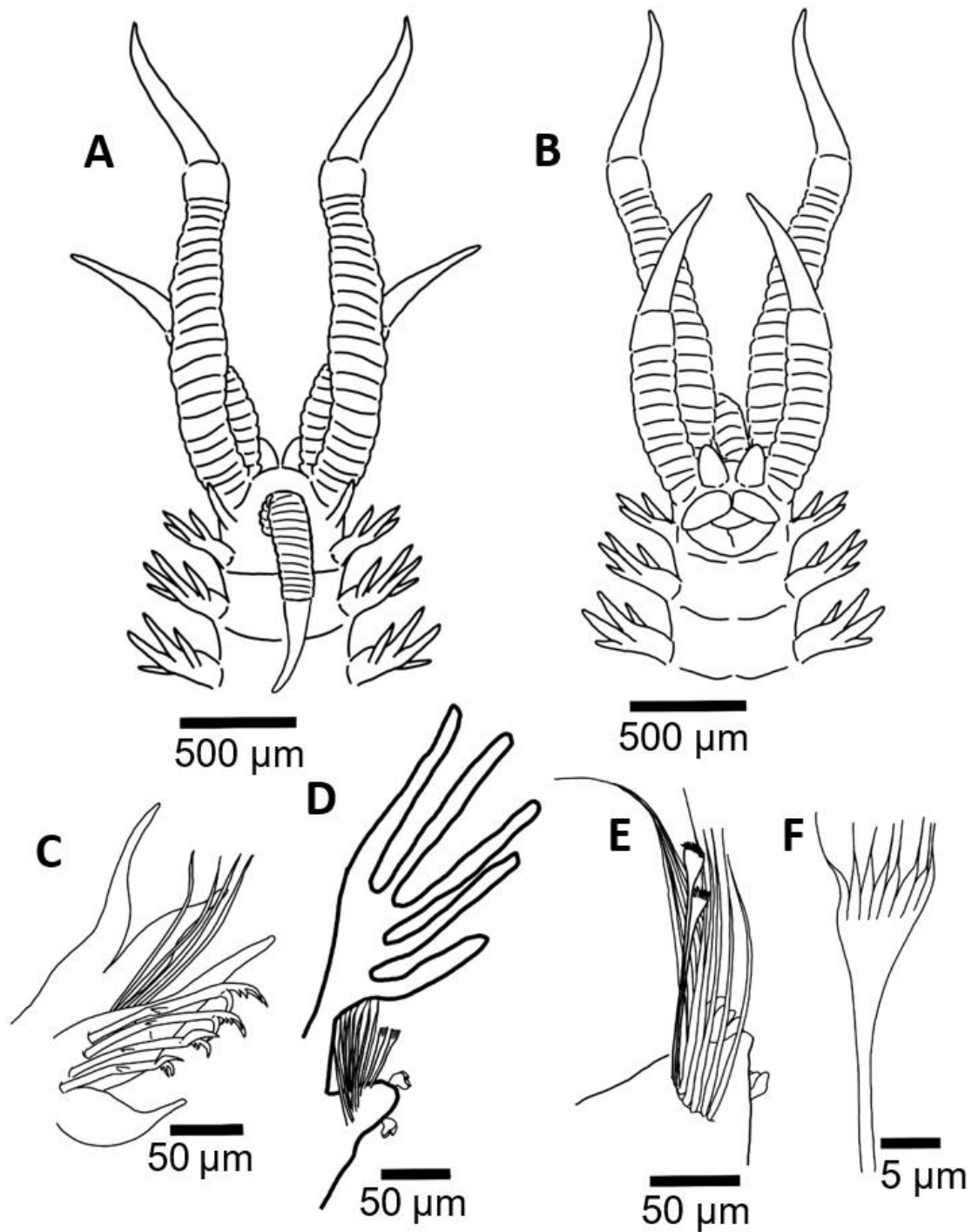


Figure 4.37: *Onuphis* sp. 2. A & B, head and antennae; C, lateral view, 1st parapodia; D, lateral view, 45th parapodia; E, lateral view of whole neuropodia and neurochaetae of the 45th parapodia; E, lateral view of two pectinate chaetae, 10 long, thin capillary neurochaetae and a pair of bidentate subacicular chaetae of 45th parapodia; F, pectinate chaetae with 8 teeth on distal end on 45th parapodia.

4.5.4.6 Family Sabellidae Latreille, 1825

Key to the Species of *Euchone* of Buntal Beach

The branchial crown with about 8 – 10 radioles, bearing 20 – 25 pinnules. Base of radioles is flattened and absence of microstylodes or macrostylodes. Collar ventrally elevated and bear a vertical incision. Thoracic neuropodial uncini are avicular in shape. Pygidium is broad and distally-rounded.....*Euchone* sp. 1

***Euchone* sp. 1**

(Figure 4.38 – Figure 4.40)

Family Sabellidae Latreille, 1825

Genus *Euchone* Malmgren, 1866

Material Examined: Paratype (2 samples): Malaysia: East Malaysia: Sarawak: Bornean water: Buntal beach, Kuching: 1°42'40' N, 110°22'35" E, 20 March 2021; 2 paratypes (catalogue no.: ASD 04278 & ASD 04279). 1 km off Buntal coast. Out of 10 specimens collected in Buntal coast, only 2 complete specimens were chosen as paratypes for detailed morphological description.

Description: The sample is about 10 – 15 mm in length and 2 mm in width, composed of 9 thoracic and approximately 70 abdominal chaetigers. Body is light pale brown after alcohol immersion. The branchial crown is about 10 mm in length with about 8 – 10 radioles. Radiolar tips medium to long, filiform, progressively tapering, distally pointed, approximately occupying the space of 20 – 25 pinnules. Base of the radioles is flattened and

absence of microstylodes or macrostylodes. The abdominal neuropodia exhibit a distinctive arrangement characterized by C-shaped compact tufts of spine-like chaetae surrounding a bundle of narrowly hooded chaetae. The trunk displays a subtle dorso-ventral flattening, gradually tapering towards the posterior end. The collar presents slightly elevated ventral margins compared to the dorsal ones, imparting an oblique appearance in lateral view. Smooth collar margins feature a small vertical incision. First chaetigers located below collar and placed at the side of the collar incision. Thoracic chaetigers present as thick structures, approximately 7 – 8 times wider than their length. Abdominal chaetigers share a similar rectangular shape but are of shorter length compared to their thoracic counterparts. Prominent and conical thoracic notopodia are observable. Thoracic notochaetae consist of a superior group of elongate narrowly hooded chaetae and an inferior group of spine-like chaetae. Thoracic neuropodial uncini are avicular in shape, featuring a short and curved neck, a well-developed breast (with the main fang not extending beyond the breast in lateral view), and a short handle. The main fang of thoracic neuropodial uncini is succeeded by two or three rows of secondary, equally sized teeth. Abdominal neurochaetae showcase a singular row of spine-like chaetae arranged in an arc pattern, enveloping a central bundle of modified elongate narrowly hooded chaetae. Posterior abdominal spine-like chaetae are narrower at the knee's region but exhibit greater length beyond the knee compared to thoracic and anterior abdominal ones. Abdominal notopodial uncini resemble those observed in the thoracic region. The pygidium is broad and distally rounded shape.

Distribution: Global distribution is unknown, in this study, *Euchone* sp. 1 was found in 3 stations (T1S3, T1S4, T2S3), which shows very fine sand to medium sand type of sediment.

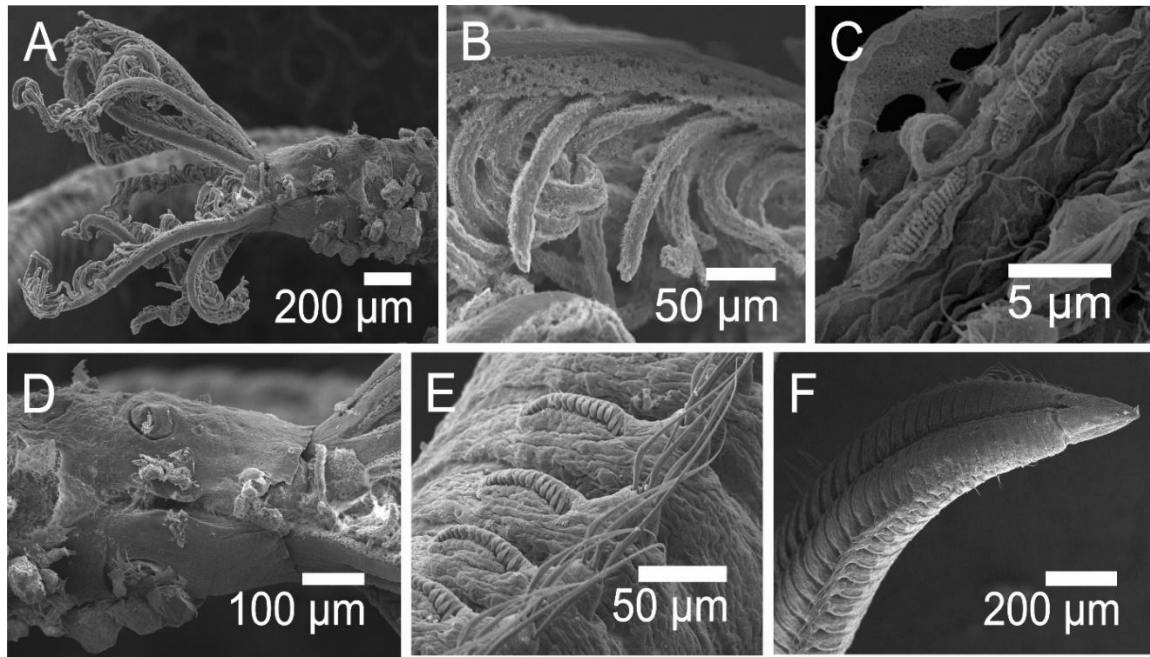


Figure 4.38: *Euchone* sp. 1. A, anterior end, dorsal view of branchial crown and peristomial collar. B, pinnules covered with sheath, attached to a radiole branch; C, the structure of sheath on the radiole and pinnules; D, vertical incision of the collar; E, thoracic neuropodial uncini on 10th segment; F, pygidium.

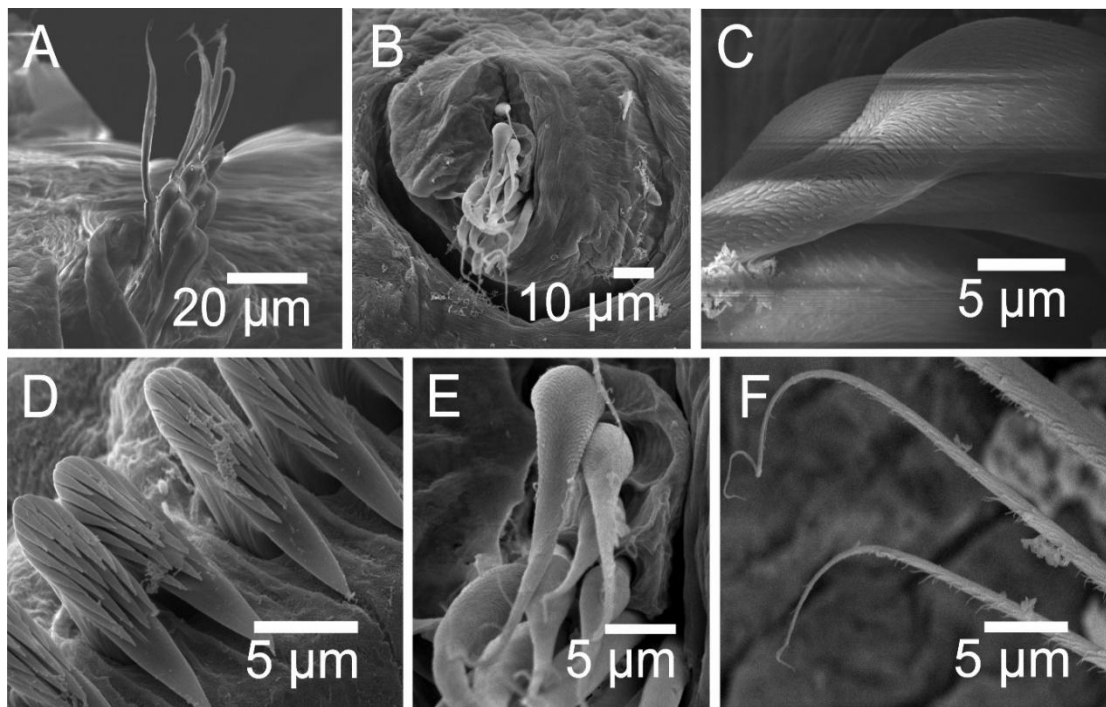


Figure 4.39: *Euchone* sp. 1. A & B, neurochaetae chaetae with thick, fleshy base that tapers to a thin end; C & E, structure of the surface of fleshy neurochaetae; D, avicular-shaped thoracic neuropodial uncini on 10th segment; F, distal end of neurochaetae, serrated capillaries.

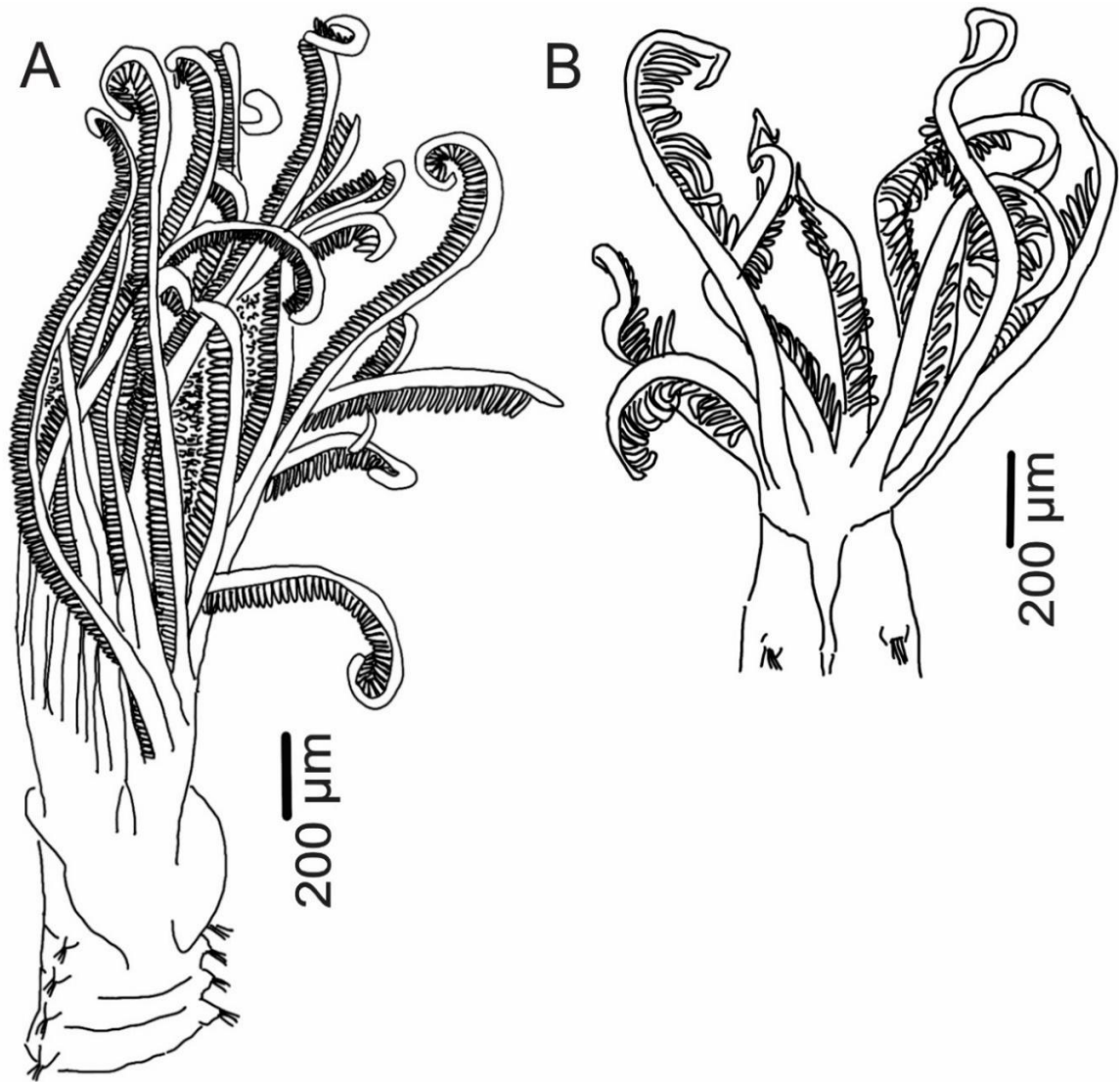


Figure 4.40: *Euchone* sp. 1. A, anterior end, lateral view of branchial crown and peristomial collar; B, dorsal view of anterior end.

4.5.4.7 Family Spionidae Grube, 1850

Key to the Species of *Scolelepis* of Buntal Beach

Palps long, extending up to chaetiger 15. Palp sheaths short, segmented, fused to base of palps. Branchiae present from chaetiger 2 to end of body, with band of cilia along inner edge of each branchiae. Pygidium with ventral cushion
..... *Scolelepis (Scolelepis) squamata* (Müller, 1806).

***Scolelepis (Scolelepis) squamata* (Müller, 1806)**

(Figure 4.41 – Figure 4.42)

Family Spionidae Grube, 1850

Genus *Scolelepis* Blainville, 1828

Material Examined: Paratype (1 sample): Malaysia: East Malaysia: Sarawak: Bornean water: Buntal beach, Kuching: 1°42'40' N, 110°22'35" E, 20 March 2021; 1 paratype (catalogue no.: ASD 04280). 1 km off Buntal coast. Out of 3 specimens collected in Buntal coast, only 1 complete specimen was chosen as paratypes for detailed morphological description

Description: The sample is 20 mm in length and 2 mm in width. Prostomium is cone-shaped, extending to a long point in the front and forming a raised, triangular shape in the back, reaching to the chaetiger 2. Palps long and thick, extending up to chaetiger 15 with cilia arranged in two bands. Palp sheaths short, segmented, fused to base of palps. Branchiae, with thick triangular tips, are present from the second segment to the end of the body, each

featuring a band of cilia along the inner edge. Parapodia of chaetiger 1 small but well-developed; have oval notopodial postchaetal lamellae and rounded neuropodial postchaetal lamellae. Both sides have capillary chaetae. Neurochaetae are in two rows on the front segments, with the neuropodia of these segments having only capillaries. The front row is broader, moderately granulated, weakly bilimbate, and shorter than the back row. Neuropodial hooded hooks from chaetiger 43, with up to 9 per neuropodium, accompanied with two long chaetae above and two short chaetae below the hooks. The hooded hooks have a blunt main fang with one or two smaller accessory teeth placed side by side and a long, slightly curved shaft. Notopodial hooded hooks from chaetiger 85 – 92, up to six per notopodium, accompanied with four long chaetae above and two short chaetae below the hooks. Pygidium with ventral cushion.

Distribution: New Caledonia, East China Sea, Andaman Sea, Sri Lanka, Persian Gulf, South Africa, Istanbul, Black Sea, Barcelona, Lisbon, North Sea, Norway, Ireland, Sweden, United Kingdom, Iceland; in this study, *Scoelepis (Scoelepis) squamata* was found in 2 stations (T1S4, T2S4), which shows very fine sand to medium sand type of sediment.

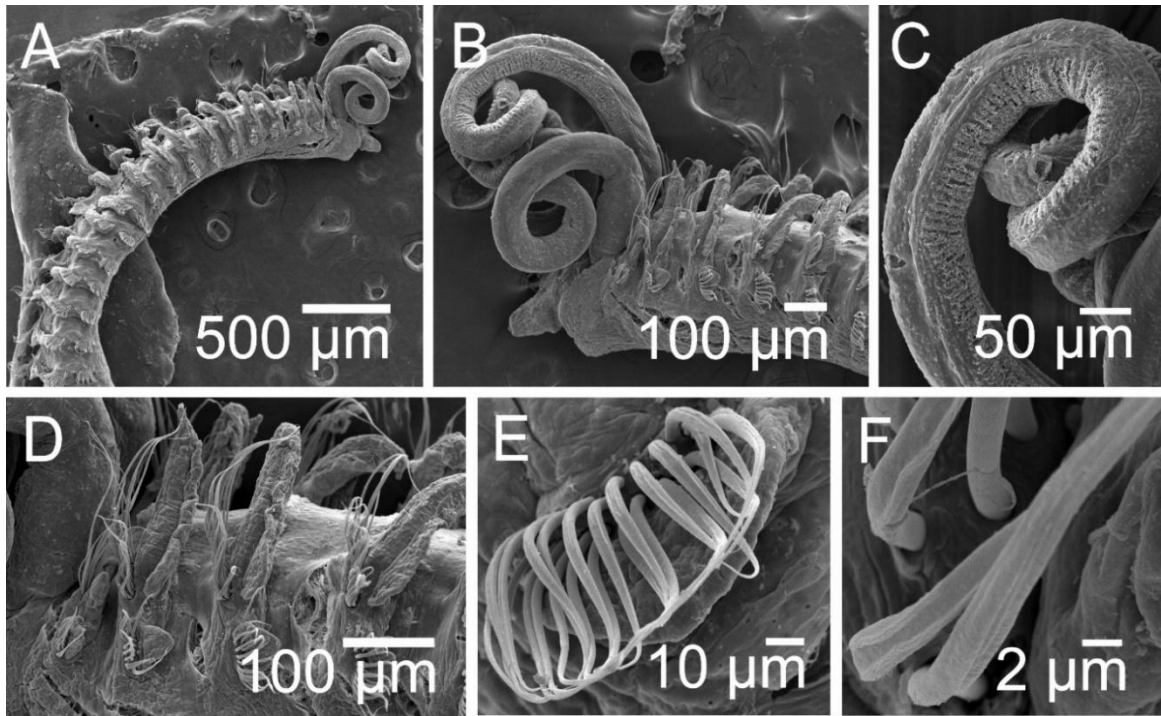


Figure 4.41: *Scolelepis (Scolelepis) squamata*. A, whole body; B, lateral view of the anterior end; C, palp with sheath on underside of the palp; D, 1st to 4th parapodia; E & F, bilimbate neurochaetae on 2nd parapodia.

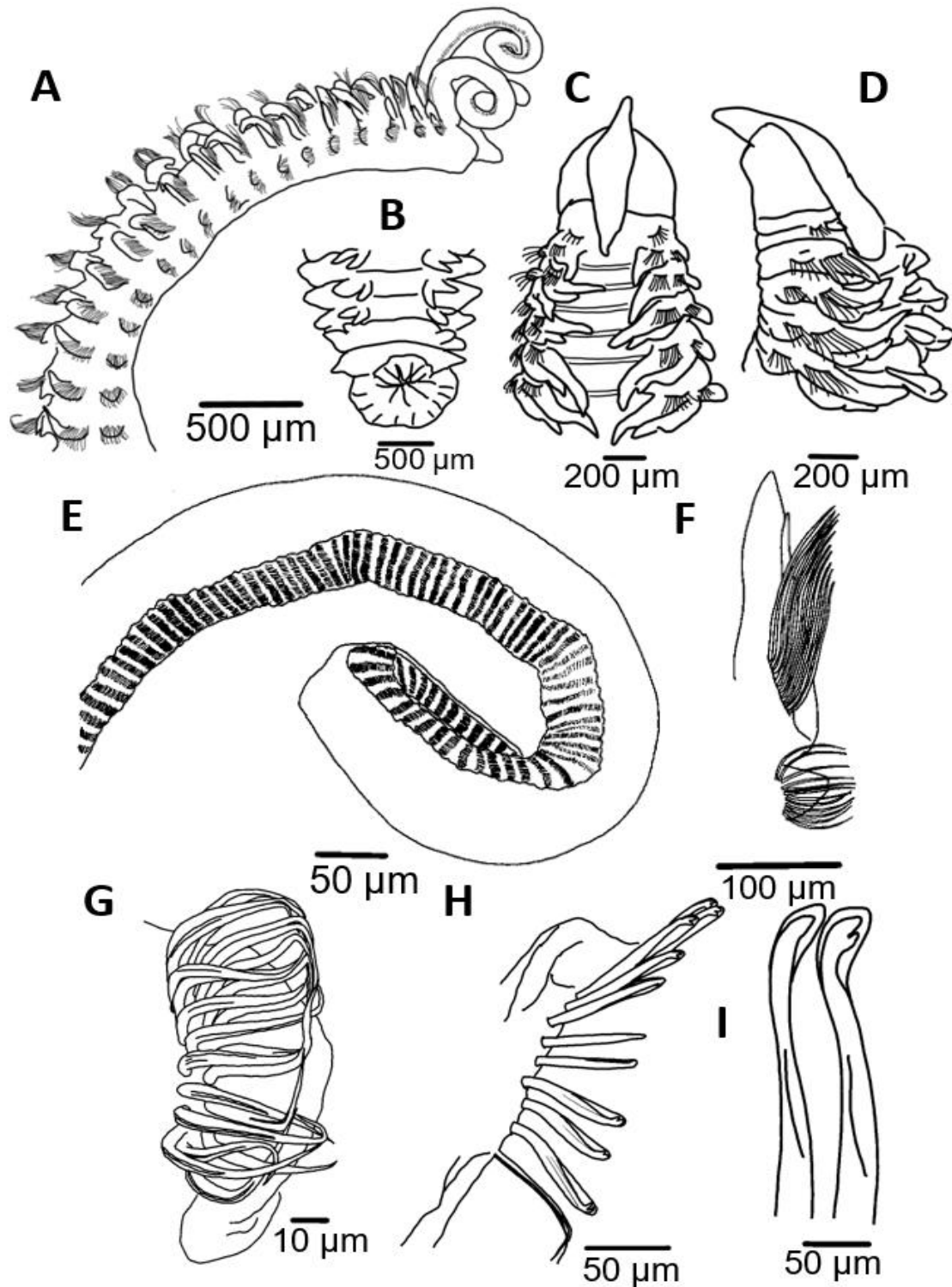


Figure 4.42: *Scolelepis (Scolelepis) squamata*. A, whole body; B, dorsal view of the pygidium; C & D, dorsal and lateral view of the anterior end; E, palp; F, 3rd parapodia; G, bilimbate neurochaetae on 3rd parapodia; H, neuropodial hooded hooks from 43rd parapodia; I, two types of neuropodial hooded hooks.

4.5.5 Polychaete Distribution and Dominancy

The observed variation in polychaete dominancy across the stations at Buntal Beach suggests localized factors influencing their distribution. Stations such as T2S2 exhibited higher abundance, potentially due to its sediment composition, as it revealed a high proportion of very fine sand/silt type of sediment. Finer sands provide a more stable substrate that facilitates burrowing and tube-building actions of polychaetes as well as efficient feeding and reproduction (Braeckman *et al.*, 2010). Polychaetes often exhibit particle size selectivity in their feeding habits, preferring the smaller organic particles present in finer sediments over larger particles found in coarser sands (Giere, 2009). This finding correlates with a similar study conducted in Buntal bay, Sarawak by Zakirah *et al.* (2019), who described grain size significantly influences community characteristics, with coarse sand supporting fewer species and lower diversity compared to finer sand. Therefore, it was observed that polychaete prefers finer sand types compared to coarser sand type due to its substrate stability and nutrient availability.

Additionally, tidal elevation was one of the major factors in influencing the abundance of polychaete in Buntal stations. T2S2 and T1S2, which located in the mid tidal zone of the Buntal beach, contributed the biggest polychaete abundance. Mid tidal elevations experience tidal cycles that provide a balance between exposure and submergence. This intermittent exposure allows polychaetes to access oxygen-rich water during flood tides while avoiding extended periods of desiccation during ebb tides (Mermillod-Blondin *et al.*, 2004). Moreover, this zone usually maintains a stable temperature and salinity conditions compared to higher elevations, which can experience more extreme fluctuations. Stable environmental conditions support physiological processes and metabolic activities of polychaetes (Gutiérrez *et al.*, 2003). In contrast, stations with <10% abundance may reflect

less favourable conditions for polychaete settlement or higher predation pressure (Vieira *et al.*, 2011). Therefore, it is believed that the higher occurrences of polychaete species in the mid tidal and low tide zone were correlated with the environmental condition during the tidal elevation of the Buntal beach.

In total, 10 species of polychaetes were identified during the study. These species belong to Family Eulepethidae, Glyceridae, Nephtyidae, Onuphidae, Sabellidae and Spionidae. Among the 6 families identified in this study, only 4 families, Glyceridae, Nephtyidae, Onuphidae and Spionidae, were found both in this study and in the research conducted by Zakirah *et al.* (2019). Among the 10 species identified in this study, only *Micronephthys sphaerocirrata* was found both in this study and in the research conducted by Zakirah *et al.* (2019). These disparities might stem from differences in the sampling timeline and sediment characteristics of each station. The sampling for this study took place in March 2020, whereas Zakirah *et al.* (2019) conducted their sampling in May 2014, indicating a significant time gap between the two studies. This discrepancy in sampling timelines may have contributed to variations in species abundance and distribution due to potential changes in environmental conditions, such as seasonal fluctuations in temperature, salinity, and nutrient availability (Smith *et al.*, 2018).

The sediment characteristics of the stations in this study predominantly consisted of fine/very fine sand, while in Zakirah *et al.* (2019), the sediment characteristics were mainly medium sand type. Sediment type is known to influence habitat suitability for various benthic organisms, as it can affect factors such as substrate stability, oxygen availability, and nutrient retention (Rhoads & Young, 1970). Therefore, the distinct sediment compositions between the two studies may have provided contrasting microhabitats, favouring different

species assemblages. In summary, the temporal gap in sampling and differences in sediment characteristics between our study and that of Zakirah *et al.* (2019) likely contributed to the observed variations in species composition.

4.5.6 Family-Specific Annotations

The polychaetes belonging to Family Onuphidae and Glyceridae are the most diverse with three species found for each family. A total of 39 individuals from Glyceridae, 31 individuals from Onuphidae, 26 individuals from Eulepethidae, 10 individuals from Sabellidae, 7 individuals from Nephtyidae, and 3 individuals from Spionidae were collected from the locations. In terms of species, *Onuphis eremita* was found the most among the species in Onuphidae family. The highest count of species among family Glyceridae is *Glycera alba* with 19 individuals collected from both transect. The most dominant species with 26 total individuals collected is *Buntalepis sarawakensis* **sp. nov.** from Family Eulepethidae, followed by *Glycera alba* from Glyceridae and *Onuphis eremita* from Onuphidae with 19 total individuals collected.

Polychaetes belonging to the Family Onuphidae, with three members of genus *Onuphis*, were found at the coast of Buntal beach. This genus is mostly represented by tubicolous scavengers and found inhabiting under and in sandy substrates, although it was observed that their occurrence was not substratum specific. The genus *Onuphis* is a cosmopolitan taxon and most of its species are characteristic for the intertidal and relatively shallow waters of warm-temperate regions (Paxton, 1986).

The Family Glyceridae, genus *Glycera*, shows a wider distribution and domination to the sampled region as this family was recorded at each sampling stations. Global records show that *Glycera* are extensively distributed from tropical to temperate regions, and from

intertidal to abyssal depths, inhabiting mainly soft bottom (sand/mud) sediments (Rizzo *et al.*, 2007; Böggemann *et al.*, 2012; Choi *et al.*, 2015; Sotomayor-Garcia *et al.*, 2020). The adaptability and resistance of this polychaete family may aid the habitation of this genus.

Other than the Onuphids, the *B. sarawakensis* **sp. nov.** was also found abundant in Buntal stations. The first record of Eulepethidae in Malaysia was *Grubeulepis malayensis* (Nishi, 2001) from Morib, Beach, near Kuala Lumpur. Other records of eulepethids from South China Sea are *Eulepethus hamifer* (Grube, 1875) from the Philippines, *Mexieulepis amioi* (Imajima, 1974) from Japan (Imajima, 1974; Pettibone, 1986), *Eulepethus nanhaiensis* (Zhang, Zhang, Osborn & Qiu, 2017) from China (Zhang *et al.*, 2017). Eulepethids were initially found from coastal regions of the Pacific, Indian and Atlantic Ocean but mainly from equatorial latitudes (Woolley & Wilson, 2011). Seven species of Eulepethidae are known to occur intertidally. Eulepethids have restricted bathymetric distributions: only four species have maximum depths in the range 400 – 800 m, the remainder occurring at depths of 80 m or shallower (Woolley & Wilson, 2011). This result indicates that the Eulepethids prefers intertidal zones and shallow areas.

B. sarawakensis **sp. nov.** are characterized by presence of a single papillae neurochaetae at the centre of the neuropodium that eventually becoming capillary towards the end of the body, presence of 1 – 2 pectinate chaetae and the segment in which the posterior lamellae first appear. This novel species possesses different morphological trait of recorded genus of Eulepethidae thus opening the probability of a new genus discovery for this species.

4.5.7 Morphological Distinction of *Buntalepis sarawakensis* sp. nov.

B. sarawakensis sp. nov. can be distinguished by other eulepethids by several features. Despite being generally similar to the genus *Grubeulepis*, these two genera can be distinguished by elytra and body segments. The elytra of *Grubeulepis* species possess fimbriated processes on its lateral borders while *B. sarawakensis* sp. nov. possess stouted fimbriated lateral processes with digitiform. The branchiae in *B. sarawakensis* sp. nov. exists in 12 pairs while branchiae in *Grubeulepis* varies between 10 – 13 pairs. The genus is also comparatively smaller in length compared to *Grubeulepis* as it only reaches up to 34 segments. *Grubeulepis* species that is slightly similar to our species is *Grubeulepis kurnai* but can be distinguished by several features. Firstly, the presence of 2 pectinate chaetae at the dorsal-most neurochaetae of segment 4 while absent in *G. kurnai*. Secondly, the elytra of *B. sarawakensis* sp. nov. possess a digitiform, frimbriated and stouted processes on its lateral segments while *G. kurnai* possess distinct lateral leaf projection between anterior and posterior surfaces.

The first species described from family Eulepethidae and genus *Grubeulepis* in Malaysia was *Grubeulepis malayensis* (Nishi, 2001). *B. sarawakensis* sp. nov. and *G. malayensis* shared the trait of having 12 pair of elytra, presence of dorsal cirri on segment 3 and 6, presence of 1 – 2 pectinate chaetae. But these species differ as *B. sarawakensis* sp. nov. have papillated first elytra, 12 pairs of branchiae starting on segments 8, 10, 12, 14, 16, 18, 20, 22, 23, 25, 26, fleshy branchiae with ventral ciliated bands, along with distally-branched cirrus, 1 pair of acicular neurochaetae, posterior lamellae start on 27th segment and presence of left and right anal cirri. *G. malayensis* have no papillated first elytra, 10 pairs of branchiae starting on segments 6, 8 10, 12, 14, 16, 18, 20, 22, 23, inflated with distal

branchial cirrus, 2 pair of acicular neurochaetae, posterior lamellae start on 26th segment and presence of a single anal cirri only.

Another species from *Grubeulepis* that possess a great similarity with our studied species is *Grubeulepis serrata* (Cutrim *et al.*, 2018) as these species shared the trait of having 12 pair of elytra with similar digitform articulations of elytra lateral processes, presence of posterior lamellae on 27th segment, notopodial capillaries with denticles and neurochaeta acicular with serrated outer edge. But these species differ as *B. sarawakensis* **sp. nov.** have 12 pairs of branchiae, hooked upper notochaetae with disciform tip and pygidium with papillated on one side at right anal cirri in while *Grubeulepis serrata* have 14 pairs of branchia, distally-rounded and knob-like hooked upper notochaete and pygidium serrated on one side at right anal cirri.

Other than that, the previous record of *Pareulepis malayana* shows a distribution of this species in South China Sea. *B. sarawakensis* **sp. nov.** can also be distinguished by presence of acicular neurochaetae on segments 3 while absent in *P. malayana*. The genus *Pareulepis* possesses notched lateral borders which is different from the *Buntalepis* **gen. nov.**, thus rejecting the probability of this species to be under the genus *Pareulepis*.

The most recent recorded Eulepethidae in South China Sea is *Eulepethus nanhaiensis* from China. *Buntalepis* **gen. nov.** is partly similar to genus *Eulepethus* but it can be distinguished by the presence of posterior lamellae. as *B. sarawakensis* **sp. nov.** possesses posterior lamellae replacing the branchiae pairs at the posterior end of the body while *E. nanhaiensis* possess numerous elytra that continuous posteriorly towards the end body, decreasing in size, with absent posterior lamellae. Compared to *E. nanhaiensis*, the presence of acicular chaetae in the neuropodia of segment 3 is tapered to a pointy and slightly hooked

end and serrated at one side on the middle to distal end margin while the acicular setae in *E. nanhaiensis* shows a conspicuous blunt-tipped and non-limbate on segment 3. Dorsal cirri on segment 3 of as *B. sarawakensis* **sp. nov.** has a more conical shape, smaller lower base and digitiform at the distal end, while segment 6 has a fleshier cirri base, more stouted and a digitiform at the distal end compared to slightly thinner, conical and absence of minute tip on dorsal cirri segment 3 of *E. nanhaiensis*. Other than that, the presence of a single papillae on the centre of the neurochaetae, laterally positioned from the middle neurochaetae but absent in *E. nanhaiensis*. The elytra of *B. sarawakensis* **sp. nov.** is fixed to 12 pairs and possess a digitiform and frimbated and stouted processes on its lateral segments while *E. nanhaiensis* have numerous pairs of elytra and possess spade-shaped processes, lateral notch or leaf-like process inside the notch. The comparison of *Buntalepis* **gen. nov.** with other Eulepethidae genus are displayed in Table 4.3.

4.6 Conclusion

Conventional morphological analysis classified polychaetes reliably into known families and species, yet struggled with identifying new species in smaller families like Eulepethidae. Firstly, these families exhibit limited morphological variation, with species often differing only in subtle features, such as setae arrangement, which are difficult to discern (George & Hartmann-Schröder, 1985). Additionally, many species lack distinct diagnostic characters, making differentiation based on morphology problematic (Fauchald, 1977). Intraspecific variation, influenced by geographic and environmental factors, complicates species identification further (Blake & Hilbig, 1994), particularly in Eulepethidae, where defining species boundaries is challenging (Pettibone, 1986). Preservation artifacts can also obscure morphological details, leading to potential misidentification (Blake & Hilbig, 1994). Moreover, the required taxonomic expertise is often lacking, particularly for less studied families, resulting in misidentifications and missed recognition of new species (Blake & Hilbig, 1994). Lastly, limited access to reference materials hampers accurate identification, as older or scarce literature on these families may not provide sufficient guidance (Blake, 2000). Further molecular analysis is crucial for accurate species identification and taxonomic placement within the phylum.

Silt and clay substrates are pivotal in sediment dynamics, providing optimal organic content and influencing polychaete distribution (Alongi, 1990). Tidal elevation also shapes their habitat, with higher elevations like the upper intertidal zone experiencing harsh conditions such as temperature fluctuations and desiccation (Levinton, 1995). Buntal beach spans 2 km from the high tide zone to the low tide zone, offering a wide intertidal region conducive to discovering various macrobenthic habitats. The beach stations predominantly feature very fine sand to sandy-silt substrates. Our study revealed higher polychaete diversity

in the mid tidal and low tidal zones compared to the high tide zones, likely due to the prevalence of silty-clay sediment in the mid-zone of Buntal beach. The discovery of *B. sarawakensis* **sp. nov.**, along with the introduction of a new genus, *Buntalepis* **gen. nov.**, from the Eulepethidae family, represents a significant scientific finding highlighting Sarawak's rich marine biodiversity. This new genus is distinguished by unique characteristics, such as elytra with a stout, fimbriae-fringed lateral border and the presence of a middle neurochaetae, setting it apart from other known taxa in the family. These findings contribute valuable insights to both local and global scientific communities, enhancing our understanding of polychaete diversity in Sarawak and emphasizing the importance of ongoing research.

CHAPTER 5

PHYLOGENETIC ANALYSIS OF *Buntalepis sarawakensis* sp. nov. (ANNELIDA: POLYCHAETA) BASED ON COI, 16S AND 28S GENES

5.1 Introduction

The preceding chapter has established the identities of the polychaetes in Buntal beach based on the morphological characteristics. In this chapter, the aim is to utilise the molecular sequence data to confirm the identity of a new polychaete species as well as infer its phylogenetic relationships. The selected DNA regions were selected as it was commonly used by taxonomist and would provide adequate reference sequences for the phylogenetic analysis. Selection of the correct evolutionary model was given great emphasis as the phylogeny inferred is heavily dependent on it. The jModel Test (Posada & Crandall, 1998) was employed to select the best evolutionary model for COI, 16S and 28S dataset. The distance (Cavalli-Sforza & Edwards, 1967; Fitch & Margoliash, 1967), parsimony (Fitch, 1971; Hartigan, 1973) and Bayesian (Huelsenbeck *et al.*, 2001) trees were built to obtain a consensus tree, confidently detailing the phylogenetic relationships of the targeted polychaete. In addition, the phylogeny inferred from COI, 16S and 28S will be compared with the previous phylogenetic report. The reference sequences employed in the phylogenetic analysis were obtained from BLAST search, thus generating the closest and furthest matches to the targeted polychaetes.

5.2 Problem Statement

Eulepethidae is a small polychaete family with only 24 recorded family since the initial described species of Eulepethidae, *Eulepethus hamifera* by Grube (1875). The lack of studies on this family also resulted in the lack of molecular data on the Eulepethidae family

in GenBank. Out of 24 recorded Eulepethidae species, only the sequences of three species (*Mexieulepis weberi*, *Grubeulepis mexicana* and *Eulepethus nanhaiensis*) are available in GenBank for phylogenetic analysis. This lack of data shows the lack of interest among polychaete researcher in this family and the lack of effort in molecular field to study its phylogeny and genetic data. The practice of molecular analysis in polychaete have been implemented worldwide but very uncommon in Malaysia.

5.3 Objectives

This study aimed to enhance the accuracy of morphological identification methods by integrating molecular analysis. Specifically, the objectives were:

- i. To determine the phylogenetic placement of the studied species, *Buntalepis sarawakensis* **sp. nov.** within the phylogenetic tree at both genus and species level among other known polychaete families
- ii. To provide essential baseline data by depositing the genetic sequences (COI, 16S and 28S genes) of the novel species, *Buntalepis sarawakensis* **sp. nov.** into GenBank for future reference.

5.4 Materials and Methods

The whole workflow of collecting, extracting and inferring phylogenetic analysis of *B. sarawakensis* **sp. nov.** using PCR sequencing of COI, 16S mitochondrial region and 28S nuclear rRNA region are simplified in the flowchart in Appendix B, Figure 2.

5.4.1 DNA Extraction Methods

5.4.1.1 DNA Extraction using CTAB Protocol (Doyle & Doyle, 1987) with some modifications

DNA samples were extracted from three individuals by using 2X CTAB (Cetyltrimethyl Ammonium Bromide) method by Doyle and Doyle (1987). Every apparatus was autoclaved and the workbench was sterilized with 70% ethanol beforehand. DNA of specimens was extracted by mincing the polychaete's whole body into 2 – 3 smaller pieces by using sterilized 22-size surgical blades and larval forceps. 200 µl of 2X CTAB buffer was added on the petri dish while mincing the sample. Another 300 µl of 2X CTAB buffer was added on the petri dish and mixed with the minced sample. The minced sample were transferred into sterilized 1.5 ml Eppendorf tube and 5 µl of Proteinase K was added into the tube. The tube was then incubated in the water bath at 60°C for an hour.

700 µl of Chloroform-Isoamyl alcohol were added into each tube and were vortexed for 10 seconds before centrifuging it for 13,000 rounds per minute (rpm) for 15 minutes at 4°C. As a result, three layers of mixtures were formed and the most upper aqueous supernatant was transferred into a new Eppendorf tube. An equal amount of cold absolute ethanol (EtOH) was added into the tube. The tube was incubated overnight at -20°C or on the bench for 20 minutes before it was centrifuged for 13,000 rpm for 15 minutes at 4°C for the second time. The EtOH was discarded and DNA pellet was observed. 500 µl of 70% EtOH was added into the tube and centrifuged at 13,000 rpm for 15 minutes at 4°C for the third time. The EtOH was discarded and pellet was observed. The tube was air dried at room temperature to remove liquid in the tube completely. Once it was completely dry, the pellet was re-dissolved in 50µl of deionized distilled water (ddH₂O) and kept in -20°C freezer for further analysis.

5.4.1.2 DNA Extraction using Qiagen DNeasy Blood & Tissue Kit

DNA samples were extracted from three individuals by using Qiagen DNeasy Blood and Tissue kit. The solution and apparatus provided in the kit were listed in Appendix C, Table 3. The sample were cut into small pieces and minced into a powder or finely divided state. This helps in breaking down the cell walls and membranes, releasing the cellular components, including DNA. The minced sample was then transferred into a 1.5 ml Eppendorf tube. 180 µl of Buffer ATL and 20 µl of Proteinase K were added into the tube. The mixture was vortexed for 15 seconds and incubated in 56°C water bath for an hour. After an hour, the mixture was taken out from the water bath and vortexed for 15 seconds.

200 µl of Buffer AL was added into the mixture and vortexed for 10 seconds. Next, 200 µl of absolute EtOH (96% – 100%) was added and vortexed for 10 seconds. 600 µl of the mixture was pipetted out and transferred into the DNeasy Mini Spin Column with collection tube provided from the kit. The spin column was then centrifuged at 8,000 rpm for 1 minute. The liquid was discarded and the collection tube was replaced. 500 µl of Buffer AW1 was added into the spin column and centrifuged at 8,000 rpm for 1 minute. The liquid was later discarded and the collection tube was replaced. 500 µl of Buffer AW2 was added into the spin column and centrifuged at 14,000 rpm for 3 minutes. The collection tube was discarded and replaced with a new 1.5 ml Eppendorf tube. 50 µl of ddH₂O was added directly into the centre of the spin column and incubated at room temperature for 1 minute to let the DNA elute. After 1 minute, the tube was centrifuged at 8,000 rpm for 1 minute to concentrate the solution. The spin column was discarded and the tube was stored in -20 °C freezer.

5.4.2 PCR Amplification

The amplification of three gene regions—COI, 16S, and 28S—was performed using the Polymerase Chain Reaction (PCR) technique. PCR was carried out in a Bio-Rad MyCycler™ Thermal Cycler (Bio-Rad Laboratories, Inc.). Each gene was amplified using specific primers, selected based on previous studies (Palumbi, 1996; Struck *et al.*, 2006; Carr *et al.*, 2011). The sequences of the oligonucleotide primers used for the PCR amplification are provided in Table 5.1. The preparation of the master mix for a single (1X) reaction is detailed in Table 5.2.

Table 5.1: List of oligonucleotide primers sequences used for PCR amplification

Targeted gene	Primer		Sequence 5' – 3'	References
COI	polyLCO1490	F	GAYTATWTTCAACAAATCATAAA GATATTGG	Carr <i>et al.</i> (2011)
	polyHCO2198	R	TAMACTTCWGGGTGACCAAARAA TCA	
16S	16S arL	F	CGCCTGTTTATCAAAAACAT	Palumbi (1996)
	16S brH	R	CCGGTCTGAACTCAGATCACGT	
28S	28F	F	CAAGTACCGTGAGGGAAAGTTG	Struck <i>et al.</i> (2006)
	PO28R4	R	G TTCACCATCTTTCGGGTCCCAAC	

Table 5.2: Preparation of master mix cocktail for one-time (1X) reaction.

Reagents	1X reaction (μL)
ddH ₂ O	16.5
GoTaq Buffer (5X)	2.8
MgCl ₂ , 25mM	1.5
dNTP, 10mM	0.5
Forward primer, 10mM	1.0
Reverse primer, 10mM	1.0
DNA template	1.5
Taq DNA polymerase, 5μ/μl	0.2

5.4.2.1 Amplification of Mitochondrial COI Region

PCR for the COI gene was performed in a 25 μ L master mix consisting of 16.5 μ L ddH₂O, 2.8 μ L of 5X GoTaq Buffer, 1.5 μ L of 25mM/L MgCl₂, 0.5 μ L of 10mM/L dNTP, 1.0 μ L of each forward and reverse COI primer adapted from Carr *et al.*, 2011 (polyLCO1490 and polyHCO2198), 0.2 μ L of 5 μ / μ L *Taq* Polymerase and 1.5 μ L DNA template. The *Taq* Polymerase DNA template were added last into the mix. The master mix was placed in PCR tubes and amplified by a repetitive series of denaturation, annealing and extension cycles in the thermocycler. For COI gene, the amplification was 94°C for 3 minutes (initial denaturation), 35 cycles of 94°C for 15 seconds (denaturation), 51°C for 30 seconds (annealing) and 72°C for 50 seconds, then continued with 72°C for 4 minutes and 4°C for infinite hold. The PCR cycling profiles for the COI primers are shown in Table 5.3.

5.4.2.2 Amplification of 16S rRNA Region

PCR for the 16S gene was performed in a 25 μ L master mix consisting of 16.5 μ L ddH₂O, 2.8 μ L of 5X GoTaq Buffer, 1.5 μ L of 25mM/L MgCl₂, 0.5 μ L of 10mM/L dNTP, 1.0 μ L of each forward and reverse 16S primer adapted from Palumbi, 1996 (16S arL and 16S brH), 0.2 μ L of 5 μ / μ L *Taq* Polymerase and 1.5 μ L DNA template. The *Taq* Polymerase DNA template were added last into the mix. The master mix was placed in PCR tubes and amplified by a repetitive series of denaturation, annealing and extension cycles in the thermocycler. For 16S gene, the amplification was 96°C for 2 minutes (initial denaturation), 35 cycles of 94°C for 30 seconds (denaturation), 48°C for 30 seconds (annealing) and 72°C for 1 minute, then continued with 72°C for 4 minutes and 4°C for infinite hold. The PCR cycling profiles for the 16S primers are shown in Table 5.3.

5.4.2.3 Amplification of 28S Nuclear rRNA Region

PCR for the 28S gene was performed in a 25 μ L master mix consisting of 16.5 μ L ddH₂O, 2.8 μ L of 5X GoTaq Buffer, 1.5 μ L of 25mM/L MgCl₂, 0.5 μ L of 10mM/L dNTP, 1.0 μ L of each forward and reverse 28S primer adapted from Struck *et al.*, 2006 (28F and PO28R4), 0.2 μ L of 5 μ / μ L *Taq* Polymerase and 1.5 μ L DNA template. The *Taq* Polymerase DNA template were added last into the mix. The master mix was placed in PCR tubes and amplified by a repetitive series of denaturation, annealing and extension cycles in the thermocycler. For S gene, the amplification was 96°C for 2 minutes (initial denaturation), 35 cycles of 94°C for 30 seconds (denaturation), 48°C for 30 seconds (annealing) and 72°C for 1 minute, then continued with 72°C for 4 minutes and 4°C for infinite hold. The PCR cycling profiles for the 28S primers are shown in Table 5.3.

Table 5.3: The PCR amplification for COI, 16S and 28S primer

Region	COI		16S and 28S	
Steps	Temperature (°C)	Cycle	Temperature (°C)	Cycle
Pre-denaturation	94°C for 3 min	1	96°C for 2 min	1
Denaturation	94°C for 15 sec	35	94°C for 30 sec	35
Annealing	51°C for 30 sec		48°C for 30 sec	
Extension	72°C for 50 sec		72°C for 60 sec	
Final Extension	72°C for 4 min	1	72°C for 4 min	1
Soaks	4°C infinite hold	1	4°C infinite hold	1

5.4.3 Visualizations of DNA Products

Visualization of DNA extractions and PCR products were done using agarose gel electrophoresis (AGE) method (Smith, 1993). 0.5g of agarose powder was weighed using a digital scale and added into 50ml of 1X Tris-Acetate-Ethylenediaminetetraacetic (TAE) buffer before microwaving in high heat for one minute. A drop of ethidium bromide (EtBr) was added into the mixture for staining purposes. Next, the mixture was poured into an electrophoresis set with comb and allowed to solidify for about 30 minutes.

5.4.3.1 AGE for Extraction Product

After the gel solidified, it was placed inside the gel box (Cleaver Scientific Ltd) and immersed in 1X TAE buffer as buffer. For DNA extraction product, a 1 kb DNA ladder was loaded into the first well to indicate the size of the yielded DNA. 1 μ L of six-time (6X) loading dye and 4 μ L of DNA template were mixed together and loaded into the well and run at 70 volts for 40 minutes. The gel was visualized with 1% agarose gel under ultraviolet (UV) transilluminator. The DNA template that indicates a clear band on the gel were selected for further PCR.

5.4.3.2 AGE for PCR Product

For the PCR products, 3 μ L of the product was loaded into the well with 100 bp DNA ladder (Fermentas GeneRuler) in the first well as the indicator of the size of the yielded product. Gel electrophoresis was run at 90 volts for 30 mins and visualized under a UV transilluminator. Photograph of the gel was taken for documentation purposes. Successful PCR products later proceeded for DNA purification.

5.4.4 Purification of Amplified Product

The amplified PCR products were subjected to a purification process using the Norgen Purification Kit. Following purification, the purified DNA was run on a 1% agarose gel and visualized using a UV transilluminator. A clear and bright DNA band indicated successful purification. The purified samples were then prepared and sent to Apical Scientific Sdn. Bhd. for sequencing. The resulting sequences were used for further molecular analysis.

5.5 Molecular Phylogenetic Analysis

Phylogenetic analysis aims to trace the evolution course of organism, in this context, polychaete, by utilizing sequences data obtained from the dominant polychaete. It is a dual pronged approach, comprising of evolutionary model selection and phylogenetic tree construction.

5.5.1 Sequence Analysis

The raw sequence obtained from the sequencing company were converted into FASTA format and pasted into notepad file to be cleaned for further analysis. BioEdit software ver. 7.2 (Hall *et al.*, 2011) program was used to reverse complement the reverse sequence and align both forward and reverse sequences. The sequence obtained from both forward and reverse were aligned and compared manually based on the chromatogram. Reverse sequences and its chromatogram were used to compare and correct the anterior end of the forward sequence and alignments were inspected and confirmed by eye and any ambiguous alignments were corrected manually in CLUSTAL X (version 2.1) (Larkin *et al.*, 2007). Notepad file containing the consensus alignment of the sequences were saved for further analysis.

5.5.2 DNA Sequencing and Basic Local Alignment Search Tool (BLAST) comparison

The consensus sequences from the selected region were submitted to BLAST (Altschul *et al.*, 1997) to search for identical and similar sequences from the Genbank database. Two BLAST algorithms were used: 1) MegaBLAST (Chen *et al.*, 2015), which was ideal for comparing a query to closely related sequences and works best if the target percent identity is 95% or more. 2) BLASTn (Altschul *et al.*, 1997) target sequences that were less similar (<95% similarity) than MegaBLAST. The 3 most significant alignments (ranked by score) from MegaBLAST were cross-checked with the morphological description for polychaete identification.

5.5.3 Sequence Alignment

To investigate the monophyly and character evolution of Eulepethidae where molecular data are so far only available for three species for the whole family, 50 additional species from were chosen based on the access to available sequences from Genbank and referring to the previously studied on phylogeny of Aphroditiformia (Gonzalez *et al.*, 2018) to obtain a stable and strong supported clade for the construction of phylogenetic tree. The majority of these sequences were also referred from Norlinder *et al.* (2012), where six members of Aphroditidae, three members of Eulepethidae and 33 additional members of Sigalionidae were included to further investigate the character evolution and relationship to the family Eulepethidae.

Based on the list of sequences used in the previous studies, species of Chrysopetalidae, Euprosinidae, Glyceridae, Nereididae, Phyllodocidae, Syllidae, and Amphinomidae were chosen as outgroups to ensure taxonomic proximities. The choice of outgroups for the Eulepethidae phylogeny study was based on their close relationship to the

ingroup, availability of molecular data, and support from previous phylogenetic studies. The selected outgroups (Chrysopetalidae, Euphrosinidae, Glyceridae, Nereididae, Phyllodocidae, Syllidae, and Amphinomidae) were phylogenetically close to Eulepethidae, had readily available sequences in GenBank, and were supported by previous research (Rouse & Fauchald, 1997; Norlinder *et al.*, 2012; Gonzalez *et al.*, 2018). This approach ensured a robust and well-supported phylogenetic tree and minimized the risk of rooting the phylogeny incorrectly, which could lead to erroneous inferences about evolutionary relationships.

For COI dataset, a total of 42 COI sequences from the Genbank nucleotide database (NCBI), inclusive of the six newly obtained COI sequences from the Buntal sample, 28 selected sequences from NCBI database taxon sampling, and eight outgroup taxa. The COI sequence of *Nereis pelagica*, *Eusyllis bloomstrandi*, *Bhawania heteroseta*, *Chrysopetalum debile*, *Glycera tridactyla*, *Chloeia flava*, *Phyllodoce maculata* and *Euphrosine foliosa* were selected as outgroup for the analyses.

For 16S dataset, a total of 48 16S sequences from the Genbank nucleotide database (NCBI), inclusive of the six newly obtained 16S sequences from the Buntal sample, 35 selected sequences from NCBI database taxon sampling, and seven outgroup taxa. The COI sequence of *Nereis pelagica*, *Eusyllis bloomstrandi*, *Bhawania heteroseta*, *Chrysopetalum debile*, *Glycera tridactyla*, *Chloeia flava* and *Euphrosine foliosa* were selected as outgroup for the analyses.

For 28S dataset, a total of 51 28S sequences from the Genbank nucleotide database (NCBI), inclusive of the six newly obtained 28S sequences from the Buntal sample, 37 selected sequences from NCBI database taxon sampling, and eight outgroup taxa. The 28S

sequence of *Nereis pelagica*, *Eusyllis bloomstrandii*, *Bhawania heteroseta*, *Chrysopetalum debile*, *Glycera tridactyla*, *Chloeia flava*, *Phyllodoce maculata* and *Euphrosine foliosa* were selected as outgroup for the analyses.

The compiled DNA sequences obtained from the Genbank for COI, 16S and 28S were aligned using MUSCLE (Edgar, 2004). The aligned sequences from MUSCLE were exported into FASTA format and later trimmed in ClustalX. Primer sequences extending the region of interest were removed to obtain a uniformed alignment. Translation of sequences into amino acid sequences was performed by using MEGA 11 (Molecular Evolutionary Genetics Analysis version 11). The nucleotide frequencies for every isolate used in the alignment (COI, 16S and 28S) was tabulated by MEGA 11. The alignment files (.mas) for both COI, 16S and 28S were exported in MEGA format (.meg) and Nexus format (.nex) for further analysis. The list of Genbank accession number for the compiled taxa sequences used in this study are shown in Table 5.4.

Table 5.4: List of taxa used throughout the phylogenetic analysis

Family	Genus	Taxon	Voucher	Collection locality	Genbank Accession no.		
					28S	16S	COI
Eulepethidae	<i>Buntalepis</i> gen. nov	<i>Buntalepis sarawakensis</i> sp. nov.	SUB12873547	Buntal Beach, Sarawak, Malaysia (Present study)	OQ458735	OQ517081	OQ517340
			SUB12913124		OQ538336	OQ517082	OQ517341
			SUB12912182		OQ538337	OQ517083	OQ517342
			SUB12906914		OQ538338	OQ517084	OQ517343
			–		OQ538339	OQ517085	OQ517344
			–		OQ538340	OQ517086	OQ517345
Aphroditidae	<i>Aproditia</i>	<i>Aphroditia aculeata</i>	SMNH118956	Gullmarsfjord, Sweden	JN852846	–	AY839578
		<i>Aphroditia negligens</i>	USNM1077212	Griffin Bay, WA	–	–	AY894309
		<i>Aphroditia</i> sp.	USNM1077213	Monterey Bay, CA	DQ790024	–	AY894310
	<i>Laetmonice</i>	<i>Laetmonice filicornis</i>	SMNH118955	Sweden	JN852847	JN852883	JN852919
	<i>Palmyra</i>	<i>Laetmonice hystrix</i>	–	Lanzarote, Canary Islands	KY823461	KY823477	KY823493
		<i>Palmyra aurifera</i>	MNHN UC92	Iles Loyaute, Pacific	–	–	AY839583
Eulepethidae	<i>Eulepethus</i>	<i>Eulepethus nanhaiensis</i>	P20150708009	South China Sea	KX587572	KX587570	KX587569
	<i>Grubeulepis</i>	<i>Grubeulepis mexicana</i>	SMNH118957	Brazil	JN852848	JN852884	–
	<i>Mexieulepis</i>	<i>Mexieulepis weberi</i>	SMNH118958	Belize	–	JN852885	JN852920
	<i>Euthalenessa</i>	<i>Euthalenessa cf. digitata</i>	–	Casamicciola, Ischia, Italy	KY657612	KY657642	–
Sigalionidae	<i>Laubierpholoe</i>	<i>Laubierpholoe</i> sp. A	–	Akumal, Mexico	–	KY823483	–
		<i>Laubierpholoe</i> cf. <i>swedmarki</i>	–	Habana, Cuba	–	KY823484	–
		<i>Laubierpholoe</i> sp. B	–	Akumal, Mexico	KY823466	KY823485	KY823499
		<i>Laubierpholoe</i> sp. C	–	Tenerife, Canary Islands	KY823467	KY823486	–
	<i>Neoleanira</i>	<i>Neoleanira tetragona</i>	SMNH118984	Trondheimsfjord, Norway/Sweden	JN852872	JN852911	AY839582
		<i>Pelagonia arenosa</i>	–	Lanzarote, Canary Islands	KY823468	KY823487	–
	<i>Pholoe</i>	<i>Pholoe baltica</i>	SMNH118985	Koster Area, Sweden	JN852873	JN85912	AY839585
		<i>Pholoe longa</i>	–	Disko Island, Greenland	KY823469	KY823488	KY823500
		<i>Pholoe minuta</i>	–	Qeqertarsuaq, Greenland	KY823470	KY823489	AY894318
		<i>Pholoe pallida</i>	SMNH118986	Trondheim, Norway	JN852874	JN852913	AY894318

	<i>Pholoe</i> sp. A	–	Napoli, Italy	KY823471	KY823490	KY823501
	<i>Pholoe</i> sp. B	–	Eilat, Israel	KY823472	KY823491	KY823502
<i>Pholoides</i>	<i>Pholoides asperus</i>	SMNH118987	California, USA	JN852875	JN852914	JN852942
<i>Pistione</i>	<i>Pistione guanche</i>	–	Tenerife, Canary Islands	KY823473	KY823492	–
	<i>Pistione guanche</i>	–	Lanzarote, Canary Islands	KY657627	KY657644	KY657661
Sigalionidae	<i>Pistione hartmannschroederiae</i>	–	Carrie Bow Cay, Belize	KY657629	KY657647	–
	<i>Pistione papuensis</i>	–	Saumarez Reef, Australia	KY657632	KY657650	KY657664
	<i>Pistione remota</i>	–	Lysekil, Sweden	KY657636	KY657654	KY657668
	<i>Pistione</i> sp. A	–	Raja Ampat, Indonesia	KY657637	KY657655	KY657669
	<i>Pistione hartmannschroederiae</i>	–	Fernando de Noronha, Brazil	KY657630	KY657648	KY657663
	<i>Pistione bulbifera</i>	–	Eilat, Israel	KY657626	KY657643	KY657660
	<i>Pistione cf. vestigialis</i>	–	Isla Bartolome, Panama	KY657640	KY657658	KY657671
	<i>Pistione puzae</i>	–	Napoli, Italy	KY657633	KY657651	KY657665
	<i>Pistione wolfi</i>	–	Caletones, Cuba	KY657638	KY657656	–
	<i>Pistione wolfi</i>	–	Trinidad, Cuba	KY657639	KY657657	KY657670
	<i>Pistonidens</i> sp.	–	Mexico	JN852876	JN852915	JN852943
	<i>Pistonidens ixazaluohae</i>	SMNH118988	Akumal, Mexico	KX282504	KX282502	KX282505
	<i>Psammoelyce</i> sp.	SMNH118989	Belize	JN852877	JN852916	–
	<i>Sigalion spinosus</i>	USNM1077225	Point Sur, CA	DQ790062	–	AY894319
	<i>Sthenelais boa</i>	USNM1077227	Brittany, France	DQ779711	DQ779635	–
	<i>Sthenelais limicola</i>	SMNH118990	Sweden	JN852878	–	–
	<i>Sthenelanelia uniformis</i>	USNM1077226	Monterey Bay, CA	DQ790064	–	–
Amphinomidae	<i>Chloeia</i>	SMNH95025	Japan	EF076781	JN852917	JN852944
	<i>Bhawanita</i>	SMNH97305	Florida, USA	EU555025	EU555044	EU555053
Chrysopetalidae	<i>Chrysopetalum debile</i>	SMNH97307	Banyuls, France	EU555027	EU555046	AF221567
	<i>Euphrosine foliosa</i>	SMNH95028	Banyuls, France	EF076785	JN852918	JN852945
Glyceridae	<i>Glyceria alba</i>	SMNH118991	Sweden	DQ779689	DQ779615	JN852946
Nereididae	<i>Nereis pelagica</i>	SMNH118992	Sweden	AY612636	AY340470	JN852947
Phyllodoceidae	<i>Phyllodoce maculata</i>	SMNH118993	Sweden	JN852879	–	AY839586
Syllidae	<i>Eusyllis blomstrandii</i>	SMNH118994	Sweden	JN852880	EF123788	EF123749

5.5.4 Phylogenetic Analysis

Phylogenetic trees constructed using Neighbour-Joining (NJ) were implemented in MEGA 11 (Saitou & Nei, 1987) while Maximum Parsimony (MP), and Maximum Likelihood (ML) were all implemented in Phylogenetic Analysis Using Parsimony (PAUP version 4.0 Beta; Swofford, 2003). Bayesian Posterior Probability (BPP) tree was constructed in MrBayes (Ronquist & Huelsenbeck, 2003). The goals of phylogenetic analyses were to determine distances between nucleotides, to emphasize the number of mutation changes, and to create a tree that gives the highest likelihood of occurring with the given data (Nakhleh *et al.*, 2005). MP, ML, and Bayesian trees were constructed with assumptions of no genetic information have transferred by hybridization in the sequences used. All of the sequences used in analysis are homologous and each position in the alignment have evolved independently. Both Maximum Parsimony (MP) and Maximum Likelihood (ML) analyses were carried out using PAUP* ver. 4.0b.10 (Swofford 2003), with the settings adhering to those specified in Teng *et al.* (2014, 2015). Pairwise genetic distances were estimated using MEGA11 (Tamura *et al.*, 2021).

5.5.4.1 Best Substitution Model Test for Evolutionary Analysis

The sequence alignment obtained from the Section 5.5.3 was subjected to evolutionary model selection by jModeltest software ver 2.1.10 (Darriba *et al.*, 2012), to determine the best evolutionary model for the aligned data set. The parameters of the best evolutionary model selected will be used to construct a maximum likelihood tree. The same parameters were also used in MrBayes v3.1.2 (Ronquist & Huelsenbeck, 2003) to build a Bayesian tree. Parsimony tree does not require any evolutionary model for construction. The aligned data were then transformed into a distance matrix with Kimura-2-parameter model

(Kimura, 1980). The Neighbour-Joining (NJ) tree was constructed using MEGA 11 (Saitou & Nei, 1987).

5.5.4.2 Maximum Likelihood Distance Tree construction (Teng *et al.*, 2014)

A distance tree utilizes DNA distance or DNA divergence computed from pairwise comparison of all isolates in an alignment. DNA distances computed using the maximum likelihood criterion with the following parameters: Substitution model followed the General-Time-Reversible model (GTR) with substitution rates (rAC, rAG, rAT, rCG, rCT, rGT) as well as base frequencies (fA, fC, fG, fT) estimated by iModelTest software ver 2.1.10. Proportion of invariable sites = 0; Variable sites = Equal rates for all sites while all other parameters were left at default. The differences between genetic distance on the tree and the pairwise distance were reduced using inverse-squared weighing (power=2). All other parameters were left at default. The bootstrap analysis was performed to justify the branching patterns generated by the distance tree. Bootstrapping was done in 1000 replicates with the Neighbour-Joining clustering method and random resampling of all aligned sites (Felsenstein, 1985). Only groups with >70% frequency were retained in bootstrap consensus tree.

5.5.4.3 Parsimony Tree construction (Teng *et al.*, 2014)

Parsimony aims to find the shortest tree (least number of nucleotide changes), hence the parameters selected aimed to find the shortest tree possible (Fitch, 1971). Nucleotide changes were assumed to be unordered (no direction of change) and delayed (DELTRAN), reducing occurrence of reversals and homoplasies. Internal node character states were limited to states observed in terminal taxa only. Multistate taxa were interpreted as uncertainties and “gap” states were treated as missing data. The most parsimonious tree (tree with the least nucleotide changes) was found using the heuristic search option (Swofford,

1996). The starting tree for branch swapping was assembled by stepwise addition with random addition of sequences in 10 different replicates. Branch swapping followed the Tree Bisection Reconnection (TBR) algorithm with the Multiple trees option (MULTREES) disabled. These random arrangements of the tree help explore a wider range of topologies, increasing the probability of finding a more parsimonious tree. The bootstrap analysis was performed in 1000 replicates with the heuristic search method and random resampling of all parsimonious informative sites. Only groups with >50% frequency were retained in the bootstrap consensus tree (Farris, 1970).

5.5.4.4 Bayesian Tree construction

The Bayesian method samples a group of trees with the highest posterior probability and calculate a consensus tree. Bayesian posterior probability (PP) was computed in MrBayes v3.1.2 (Ronquist & Huelsenbeck, 2003) using the evolutionary model estimated by the Best Model Analysis as well as the prior probabilities of the parameters involved. Trees with the highest posterior probabilities were sampled by Metropolis-couple Markov Chain Monte Carlo (MCMCMC) from 4 chains of 12,000,000 generations. All other parameters were left at default. A total of 120,001 trees were sampled from the posterior probability distribution (one every 100 generations) and the initial 50% or 60,000 trees were discarded as “burn-in” to ensure the chains have reached stationarity. For this analysis, BI was selected as the consensus tree for its statistical rigor, ability to handle complex models, and provision of meaningful uncertainty measures, ensuring more reliable phylogenetic results (Huelsenbeck & Ronquist, 2001; Ronquist & Huelsenbeck, 2003; Lartillot & Philippe, 2004; Drummond & Rambaut, 2007).

5.6 Results and Discussion

5.6.1 Sequence Analysis

5.6.1.1 *Buntalepis sarawakensis* sp. nov. sequences

The newly generated sequences of *Buntalepis sarawakensis* sp. nov. were submitted to Genbank for accession number registration. The partial sequence analysis for the COI, 16S and 28S genes of the six Buntal samples were tabulated in Table 5.5.

Table 5.5: The sequence analysis of the six Buntal sample

Buntal sample (<i>Buntalepis sarawakensis</i> sp. nov.)			
Targeted gene	COI	16S	28S
Sequence length	504 – 507	479	717 – 722
Final alignment	507 bp	479 bp	722 bp
Gaps	No gaps	No gaps	6 sites
BLAST result	83% similar to <i>Eulepethus nanhaiensis</i> (Family Eulepethidae)	88% similar to <i>Mexieulepis weberi</i> (Family Eulepethidae)	81% similar to <i>Grubeulepis mexicana</i> (Family Eulepethidae)
Registered	OQ517340	OQ517081	OQ458735
GenBank	OQ517341	OQ517082	OQ538336
Accession No.	OQ517342	OQ517083	OQ538337
	OQ517343	OQ517084	OQ538338
	OQ517344	OQ517085	OQ538339
	OQ517345	OQ517086	OQ538340

BLAST results identified the polychaete at the family level only, indicating low similarity for genus classification. Phylogenetic tree was constructed to further confirm its placement in the Eulepethidae family using mitochondrial (COI, 16S) and ribosomal (28S) genes.

5.6.1.2 Compilation sequences

A total of 50 additional sequences were compiled and aligned with the Buntal sample for the construction of the phylogenetic tree. The sequence analysis for the compilation of taxa sequences from Genbank and Buntal sample were tabulated in Table 5.6.

Table 5.6: The sequence analysis of the compilation sequences from Genbank and Buntal sample

Compilation of taxa sequences from Genbank and Buntal sample			
Targeted gene	COI	16S	28S
Number of sequences aligned	42 sequences	48 sequences	51 sequences
Final alignment	568 bp	521 bp	680 bp
Number of gaps	1 site	56 sites	81 sites

The 568 bp nucleotides of COI final compiled sequence length comprised of 224 conserved sites, 343 variable sites, 271 parsimony-informative and 72 singleton variable sites. The 521 bp nucleotides of 16S final compiled sequence length comprised of 143 conserved sites, 374 variable sites, 324 parsimony-informative and 45 singleton variable sites. The 680 bp nucleotides of 28S final compiled sequence length, comprised of 253 conserved sites, 388 variable sites, 297 parsimony-informative and 85 singleton variable sites.

5.6.2 Phylogeny

Figure 5.1 to 5.3 show phylogenetic trees from COI, 16S, and 28S gene analysis of *Buntalepis sarawakensis* **sp. nov.** with other polychaetes. The BI tree was used as consensus tree to integrate results from NJ, MP, ML, and BI methods.

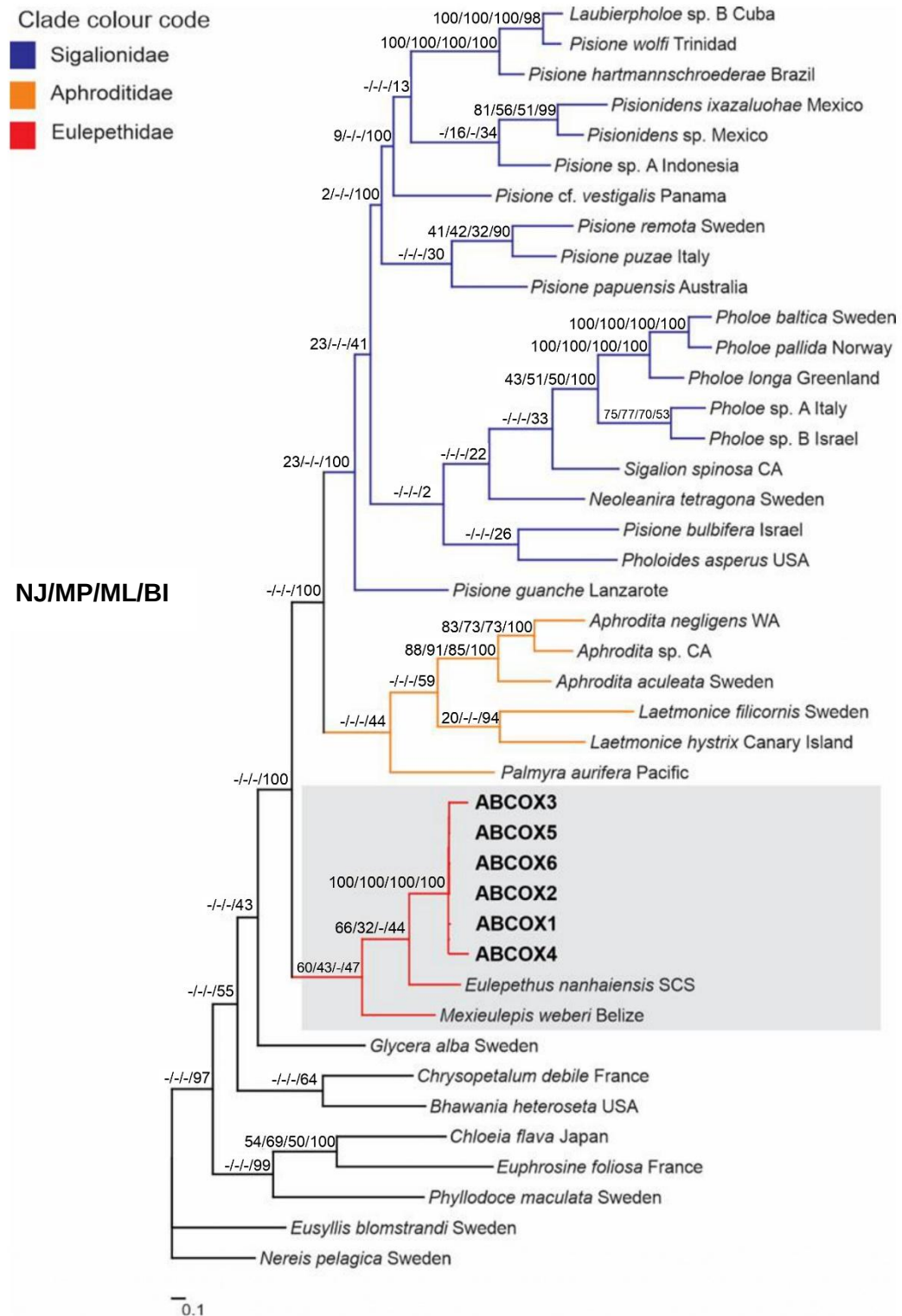


Figure 5.1: The Bayesian Posterior Probability (BPP) values for COI using Neighbour-Joining (NJ), Maximum Parsimony (MP), Maximum Likelihood (ML), and Bayesian Inference (BI) methods, with 3 major clades and rooted to *Nereis pelagica* Sweden as outgroup. Numbers on the branches indicate bootstrap and posterior probability values for NJ/MP/ML/BI tree. The acronym ABCOX signifies isolates from Buntal. Dash (-) indicate unavailable or >0.5 bootstrap value.

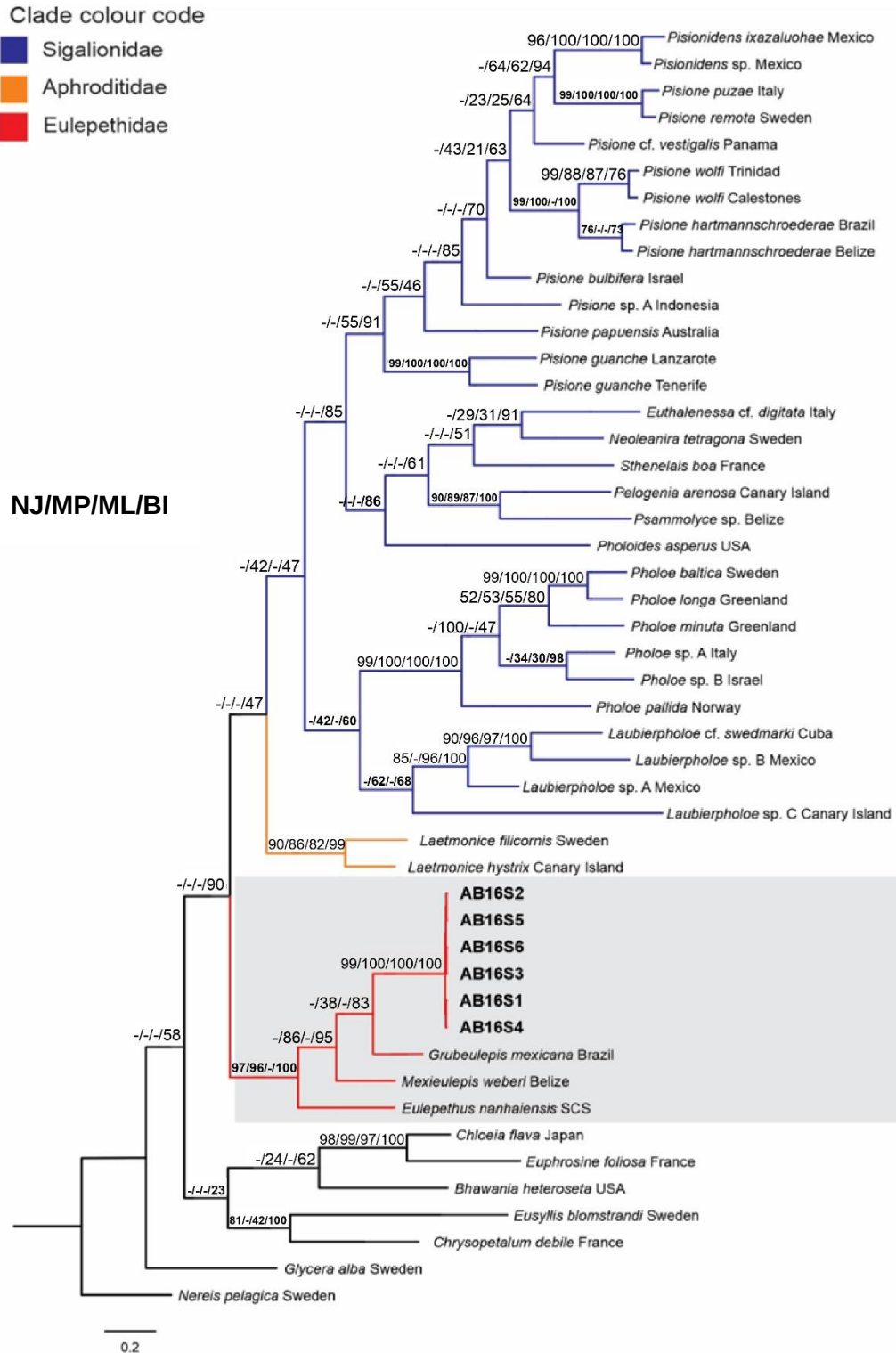


Figure 5.2: The Bayesian Posterior Probability (BPP) values for 16S using Neighbour-Joining (NJ), Maximum Parsimony (MP), Maximum Likelihood (ML), and Bayesian Inference (BI) methods, with 3 major clades and rooted to *Nereis pelagica* Sweden as outgroup. Numbers on the branches indicate bootstrap and posterior probability values for NJ/MP/ML/BI tree. The acronym AB16S signifies isolates from Buntal. Dash (-) indicate unavailable or >0.5 bootstrap value.

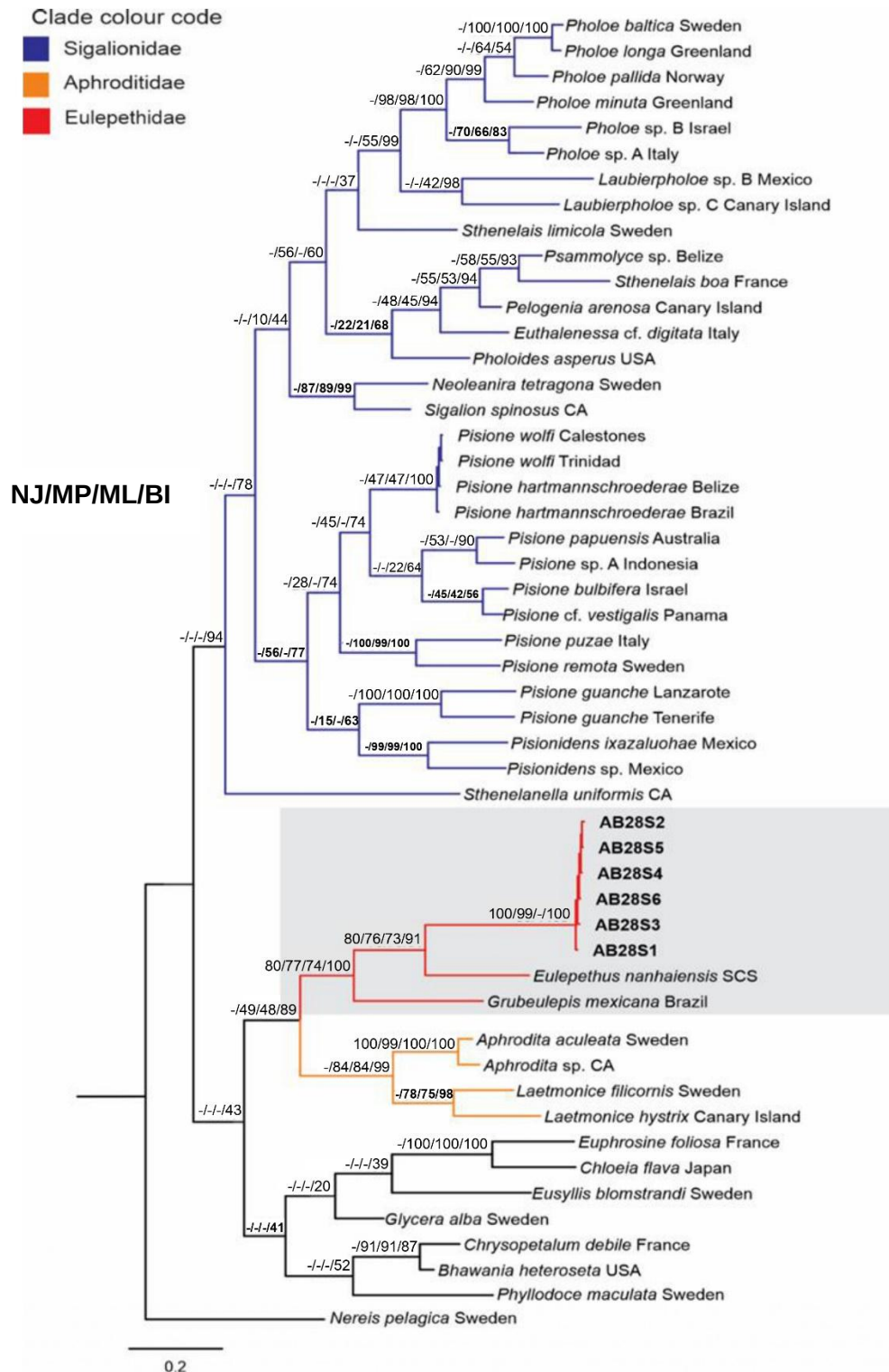


Figure 5.3: The Bayesian Posterior Probability (BPP) values for 28S using Neighbour-Joining (NJ), Maximum Parsimony (MP), Maximum Likelihood (ML), and Bayesian Inference (BI) methods, with 3 major clades and rooted to *Nereis pelagica* Sweden as outgroup. Numbers on the branches indicate bootstrap and posterior probability values for NJ/MP/ML/BI tree. The acronym AB28S signifies isolates from Buntal. Dash (-) indicate unavailable or >0.5 bootstrap value.

5.6.2.1 COI sequence analysis

Analysis of the 6 individuals of *B. sarawakensis* **sp. nov.** for COI sequences demonstrated that the six isolates had identical sequences, representing a single species. In the COI tree (Figure 5.1), *B. sarawakensis* **sp. nov.** clustered with *E. nanhaiensis* SCS with weak nodal support (NJ, 66%; MP, 32%; ML, <50%; BI, 44%) and formed a sister taxon to *M. weberi* Belize with no strong nodal support (NJ, 60%; MP, 43%; ML, <50%; BI, 47%). The COI gene sequence of this species was unique to its own species compared to its related organisms and their common ancestors. Therefore, the lack of synapomorphy prevented the inference of signature sequence of COI gene that matches the pairwise of *E. nanhaiensis* SCS and *M. weberi* Belize. A confirmatory test using Blastn search showed matches of *E. nanhaiensis* SCS or *M. weberi* Belize only when using its full nucleotide sequence, with all showing E-values of 10^{-177} (Blastn search performed on 25th January, 2024). Due to the lack of COI sequences for *Grubeulepis mexicana* from the Genbank, only two sequences of *Eulepethus nanhaiensis* SCS and *Mexieulepis weberi* Belize were used for the COI analysis in this study. The average pairwise distance and sequence divergence of COI sequences between *B. sarawakensis* **sp. nov.** and Eulepethidae members were displayed in Table 5.7.

From these analyses, the NJ, MP, ML and BI trees made it clear that the six isolates were classified under the Eulepethidae family, but formed a well-separated lineage from the neighbouring genera, *Eulepethus* and *Mexieulepis*. This confirms the presence of monophyletic clade that was phylogenetically distinct from any recognized genera in Eulepethidae, providing high level support (100% bootstrap support) for considering them as a distinct genus. Therefore, the COI tree indicates that *B. sarawakensis* **sp. nov.** was placed in the Eulepethidae family, but not assigned under any recorded genus in Eulepethidae and were perceived as a new sister genus to *Eulepethus* and *Mexieulepis*. The

average pairwise distance between *B. sarawakensis* **sp. nov.** and Eulepethidae members were 20%. The sequence divergence with *E. nanhaiensis* SCS and *M. weberi* were displayed in Table 5.7.

5.6.2.2 16S sequence analysis

Analysis of the 6 individuals of *B. sarawakensis* **sp. nov.** for 16S sequences demonstrated that the six isolates had identical sequences, representing a single species. The 16S tree (Figure 5.2) shows the clustering of *B. sarawakensis* **sp. nov.** with *G. mexicana* Brazil but with weak nodal support (NJ, <50%; MP, 38%; ML, <50%; BI, 83%) and formed sister taxa to *E. nanhaiensis* and *M. weberi* but with strong nodal support (NJ, <50%; MP, 86%; ML, <50%; BI, 95%) and (NJ, 97%; MP, 96%, ML, <50%; BI, 100%). The molecular signature of 16S gene with the synapomorphy of 5'- AAC GGC CGC GGT ATC CTG ACC GTG CGA AGG TAG CAT AAT CAT TTG CCT CTT AAT TGG TGG CTA GAA TGA ATG GAC AAA CGA GAT TA-3' (86 bp), is located on the nucleotides 51 – 136 in the DNA sequence. The signature sequence includes five single nucleotide polymorphisms (SNPs) when compared pairwise to *E. nanhaiensis* SCS, *G. mexicana* Brazil and *M. weberi* Belize. A confirmatory test using Blastn search showed that the first four matches were to sequences of *M. weberi* Belize, *E. nanhaiensis* SCS and *G. mexicana* Brazil, with showing E-values of 10^{-33} (Blastn search performed on 25th January, 2024). Three sequences from the Eulepethidae family, *Eulepethus nanhaiensis* SCS, *Grubeulepis mexicana* Brazil and *Mexieulepis weberi* Beliza were used for the 16S analysis in this study.

From these analyses, the NJ, MP, ML and BI trees made it clear that the six isolates were classified under the Eulepethidae family, but formed a well-separated lineage from the neighbouring genera, *Eulepethus*, *Grubeulepis* and *Mexieulepis*, with it being the closest to

G. mexicana Brazil (83% bootstrap value). This confirms the presence of monophyletic clade that was phylogenetically distinct from any recognized genera in Eulepethidae, providing high level support (99%-100% bootstrap support) for considering them as a distinct genus. Therefore, the 16S tree also indicates that *B. sarawakensis* **sp. nov.** was placed in the Eulepethidae family, but not assigned under any recorded genus in Eulepethidae and were perceived as a new sister genus to *Eulepethus*, *Grubeulepis* and *Mexieulepis*. The average pairwise distance and sequence divergence of 16S sequences between *B. sarawakensis* **sp. nov.** and Eulepethidae members were displayed in Table 5.7.

5.6.2.3 28S sequence analysis

Analysis of the 6 individuals of *B. sarawakensis* **sp. nov.** for 28S sequences demonstrated that the six isolates had identical sequences, representing a single species. The 28S tree (Figure 5.3) revealed a clustering of *B. sarawakensis* **sp. nov.** with *E. nanhaiensis* similar to that of the COI tree, but with stronger nodal support (NJ, 80%; MP, 99%; ML, <50%; BI, 100%), and formed a sister taxon to *G. mexicana* with strong nodal support (NJ, 80%; MP, 77%; ML, 74%; BI, 100%). The molecular signature of 28S rDNA gene with the synapomorphy of 5'-GTG GCG AAT CGG TCG GCA CCC CAT CCG ACC CGT CTT GAA ACA CGG ACC AAG GAG TCT AAC ATG TGC GCG AGT CAT GGG G-3' (79 bp), is located on the nucleotides 509 – 587 in the DNA sequence. The signature sequence completely matches the pairwise of *Eulepethus nanhaiensis* and *Grubeulepis mexicana* with no indel or single nucleotide polymorphism. A confirmatory test using Blastn search showed that the first two matches were to sequences of *Eulepethus nanhaiensis* and the ninth match of *Grubeulepis mexicana*, with all showing E-values of 10^{-31} (Blastn search performed on 25th January, 2024). Due to the lack of 28S sequences for *Mexieulepis weberi* from the

Genbank, only two sequences of *Eulepethus nanhaiensis* SCS and *Grubeulepis mexicana* Brazil were used for the 28S analysis in this study.

From these analyses, the NJ, MP, ML and BI trees indicated that the six isolates were classified under the Eulepethidae family, but formed a well-separated lineage from the neighbouring genera, *Eulepethus* and *Mexieulepis*, with it being the closest to *E. nanhaiensis* SCS (73%-91% bootstrap value). This confirms the presence of monophyletic clade that was phylogenetically distinct from any recognized genera in Eulepethidae, providing high level support (100% bootstrap support) for considering them as a distinct genus. Therefore, the 28S tree indicates that *B. sarawakensis* **sp. nov.** was placed in the Eulepethidae family, but not assigned under any recorded genus in Eulepethidae and were perceived as a new sister genus to *Eulepethus* and *Mexieulepis*. The average pairwise distance and sequence divergence of 28S sequences between *B. sarawakensis* **sp. nov.** and Eulepethidae members were displayed in Table 5.7.

5.6.2.4 Sequence divergence

B. sarawakensis **sp. nov.** showed varying levels of sequence divergence when compared to closely related species across different genetic markers.

Table 5.7: The average pairwise distance and sequence divergence of *Buntalepis sarawakensis* **sp. nov.** and the Eulepethidae family

Genetic Marker	Average Pairwise Distance with Eulepethidae (%)	Species	Sequence Divergence (%)
COI	20%	<i>Eulepethus nanhaiensis</i>	19% - 20%
		<i>Mexieulepis weberi</i>	23% - 24%
16S	14%	<i>Eulepethus nanhaiensis</i>	13% - 14%
		<i>Grubeulepis mexicana</i>	10% - 13%
		<i>Mexieulepis weberi</i>	10% - 12%
28S	26%	<i>Eulepethus nanhaiensis</i>	20% - 21%
		<i>Grubeulepis mexicana</i>	21% - 22%

The relatively low average pairwise distances, especially in the COI (20%) and 16S (14%) datasets, indicate a shorter genetic distance between *B. sarawakensis* **sp. nov.** and Eulepethidae members, suggesting close evolutionary relationships within this clade. In COI and 28S BI consensus tree, *B. sarawakensis* **sp. nov.** were placed adjacent to *E. nanhaiensis* with high support level (91%) in 28S consensus tree. While in 16S consensus tree, the closest member to *B. sarawakensis* **sp. nov.** was *G. mexicana* as it is also supported with high bootstrap value (83%) and followed by *M. weberi* (95%) and *E. nanhaiensis* (100%). Considering that the BLASTn result for COI and 28S sequences result also showed similarities (>80%) to *E. nanhaiensis*, it is suggested that as *B. sarawakensis* **sp. nov.** might be associated genetically to the *Eulepethus* genera but not too close for them to be considered in the same genera.

5.7 Conclusion

For this study, molecular sequence data has aided the identification and classification of as *B. sarawakensis* **sp. nov.** collected from Buntal beach. The COI gene, with its high resolution at the species level, combined with the broader taxonomic insights offered by the 16S and 28S rRNA genes, enables a comprehensive view of the phylogenetic structure. By integrating these three genetic markers, species boundaries and deeper phylogenetic relationships can be clarified within polychaetes.

The uniqueness of the COI gene of this species has made the inference of signature sequences for COI gene on this species challenging, possibly due to high intraspecific variability and limited reference sequences. In some species, significant variation within the COI gene makes it difficult to distinguish species based on this marker alone, especially when morphological differences are subtle or absent (Nygren, 2014). The scarcity of COI reference sequences in public databases for many polychaetes adds to this challenge, as fewer comparative sequences are available for accurate species identification (Carr *et al.*, 2011). To overcome these challenges, a multi-gene approach, combining mitochondrial (e.g., COI, 16S) and nuclear markers (e.g., 28S), is essential for more accurate species identification and phylogenetic resolution (Colgan *et al.*, 2006). Furthermore, expanded sampling of underrepresented species and the development of more sophisticated primers that avoid pseudogene amplification can improve the accuracy of COI-based studies (Song *et al.*, 2008). These strategies, along with a more thorough investigation of polychaete diversity, could significantly enhance our understanding of the evolutionary relationships within this group.

B. sarawakensis **sp. nov.** were distributed in Eulepethidae clade, signifies its closeness with the members in this family. *B. sarawakensis* **sp. nov.** identified in Eulepethidae clade of all trees also formed a well-supported branch from the same ancestors. The trees showed a consistent grouping of *B. sarawakensis* **sp. nov.** with other members in this family such as *E. nanhaiensis* SCS, *G. mexicana* Brazil and *M. weberi* Belize. The branching patterns and high bootstrap values observed in individual major clades suggests the polychaetes within these clades to be closely related.

The phylogenetic analysis supported the morphological observations that the Buntal isolates are a new species, herein described as *B. sarawakensis* **sp. nov.** Phylogenetically, *B. sarawakensis* **sp. nov.** clustered with its sister taxa (*E. nanhaiensis* + *M. weberi* + *G. mexicana*) in the COI, 16S and 28S consensus tree. Morphologically, *B. sarawakensis* **sp. nov.** showed more morphometric overlap with the *Grubeulepis* species. However, despite sharing the same average of body segment, number of elytra pair, presence of fimbriated lateral elytra processes and presence of posterior lamellae, *B. sarawakensis* **sp. nov.** possess multiple distinguishing features from the *Grubeulepis* genus such as the presence a distinct middle neurochaete on all parapodia and presence of posterior on the the 27th segment. The morphological overlap with *Grubeulepis* species alongside phylogenetic clustering with distinct sister taxa suggests an evolutionary divergence within the new genus. The discovery of *B. sarawakensis* **sp. nov.** not only contributes to the understanding of local biodiversity but also underscores the need for further taxonomic and genetic studies to elucidate its genetic characteristics, ecological roles and adaptations within its habitat.

CHAPTER 6

GENERAL CONCLUSION AND RECOMMENDATIONS

The study of polychaete habitats at Buntal Beach has confirmed its suitability for a diverse range of polychaete families, particularly Eulepethidae, Glyceridae, and Onuphidae. The silty-clay substrate at this location exhibits a dominance of polychaetes, which facilitates their growth and survival. Through the comprehensive identification of 10 species, including a newly discovered genus and species, this study significantly contributes to the documentation of marine biodiversity in Sarawak. This documentation is essential for baseline data on polychaete identification, distribution and serving as a critical reference for potential threats to polychaete populations.

Conventional identification analysis through morphological comparison has proven reliable and valid for detecting and identifying previously recorded species. However, this method is limited in its ability to identify unrecorded, new, or cryptic species. Therefore, it is essential to employ advanced molecular analysis methods to avoid misidentifications of each species. Molecular techniques are crucial in modern taxonomy and species identification, but conventional morphological methods remain valuable for their tangible and visual characterization of biological diversity. Integrating both conventional and molecular approaches often provides a comprehensive and accurate understanding of species identification and classification.

The Eulepethidae family are severely understudied as only 6 known genus and a cumulative of 24 total species under this family. From a total of 24 species recorded under Eulepethidae family, only three of the species; *E. nanhaiensis*, *G. mexicana* and *M. weberi*,

have their molecular sequence available in BLAST, indicating a very low interest for molecular research in this species. The available sequence data available in BLAST for the Eulepethidae family are very limited, thus making it difficult to determine the placement of this species in any genus or species. Based on its phylogeny data, *B. sarawakensis* **sp. nov.** might be associated nearby to the *Eulepethus* genera and a sister clade for *Eulepethus*, *Grubeuelpis* and *Mexieulepis*. However, the lack of COI sequence sample of *G. mexicana* and 28S sequence sample of *M. weberi* available are making it impossible to determine which one of the genera is assigned closer to *B. sarawakensis* **sp. nov.** without further molecular research and additional detailed research of this family.

Certain polychaete species, if abundant and amenable to cultivate, show great potential as a valuable feed source in aquaculture. Moreover, polychaetes are widely applied as bait in commercial fisheries. Exploring into the intricacies of specific families, like Eulepethidae, not only enriches our comprehension of polychaete diversity, ecology, and evolution but also unveils unique insights into their distinctive characteristics and ecological roles. Expanding research on this family is crucial due to the limited knowledge and potential benefits it could offer. Continued exploration could unlock its potential in aquaculture, improve commercial fishing practices, and reveal new opportunities similar to other known polychaete species.

The initial attempt to utilize a universal primer for this particular species resulted in unamplified and failure in sample sequencing. This challenge led to the innovation of using a previously modified primers by Carr *et al.* (2011), Palumbi (1996) and Struck *et al.* (2006), ultimately enabling the successful sequencing of the novel *B. sarawakensis* **sp. nov.** Recognizing the importance of consistent primer performance across all taxa, there emerges

a compelling call to develop primers that reliably serve as an identification tool. The methods and findings unveiled in this research addressed a crucial gap in knowledge regarding the availability of a suitable universal primer and set a promising precedent for forthcoming studies in similarly underexplored regions.

In conclusion, the study conducted at Buntal Beach has highlighted its suitability as a habitat for various polychaete families, notably Eulepethidae, Glyceridae, and Onuphidae, thriving in the silty-clay substratum. The comprehensive identification of 10 species, including a newly discovered genus and species, through the incorporation of advanced molecular techniques alongside conventional morphological analyses enhances our ability to accurately identify and classify species. Due to the underrepresentation of molecular data for the Eulepethidae family, pending further research on the placement of *B. sarawakensis* **sp. nov.** among the Eulepethidae species are needed to clarify its exact relationships.

Several future study recommendations can significantly advance the understanding of polychaete taxonomy and molecular biology. Conducting comprehensive field surveys across diverse geographic regions and habitats can help establish a repository of polychaete specimens. By sampling species from diverse marine environments like coastal areas, deep-sea habitats, and estuaries, researchers can create a comprehensive database that accurately reflects global polychaete biodiversity. This database serves as a crucial resource for ongoing and future research in polychaete taxonomy and distribution.

Expanding the molecular database for Eulepethidae species and conducting targeted studies to sequence additional genes, such as cytochrome B or ITS genes, will be fundamental. Detailed phylogenetic analyses using a combination of mitochondrial and nuclear markers can illuminate evolutionary relationships within and between polychaete

families. This approach not only clarifies the placement of new species like *B. sarawakensis* **sp. nov.** but also enriches genetic databases, enhancing the accuracy of phylogenetic analyses and species identification.

Moreover, future research should prioritize the development and validation of new primers tailored specifically to amplify genetic material from understudied polychaete families like Eulepethidae. This initiative can overcome current challenges with universal primers and enhance the reliability of molecular identification techniques. Furthermore, establishing a standardized protocol for DNA extraction, amplification, and sequencing specific to polychaetes is crucial. This protocol will ensure consistency and reproducibility across studies, providing essential guidelines for molecular workflows that mitigate contamination risks and maintain the precision of genetic data.

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APPENDICES

Appendix A: Sampling Method

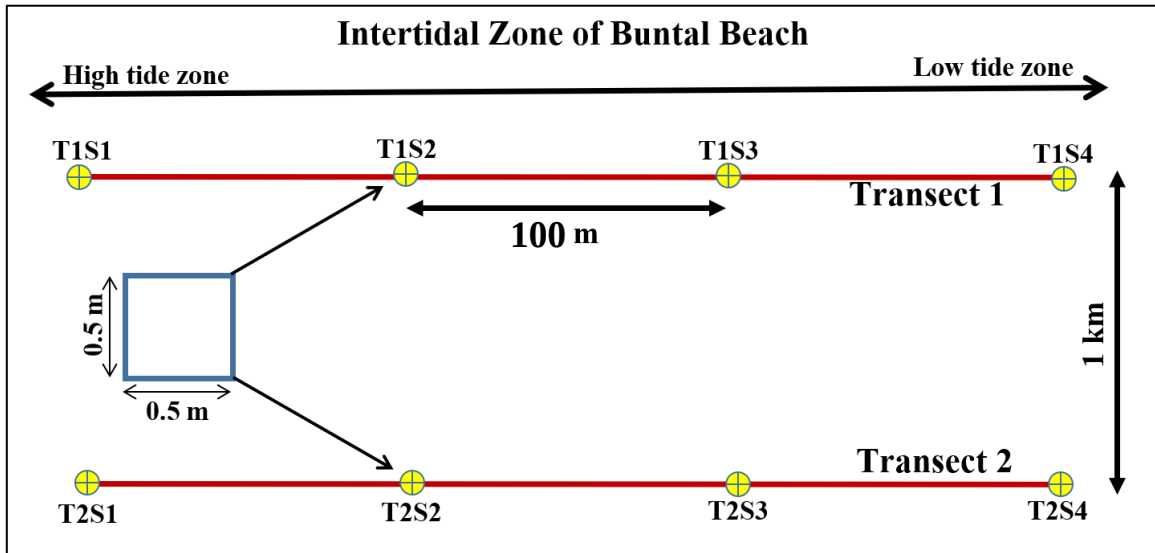


Figure 1: Illustration of sampling stations from Buntal beach selected in this study

Appendix B: Scanning Electron Microscope

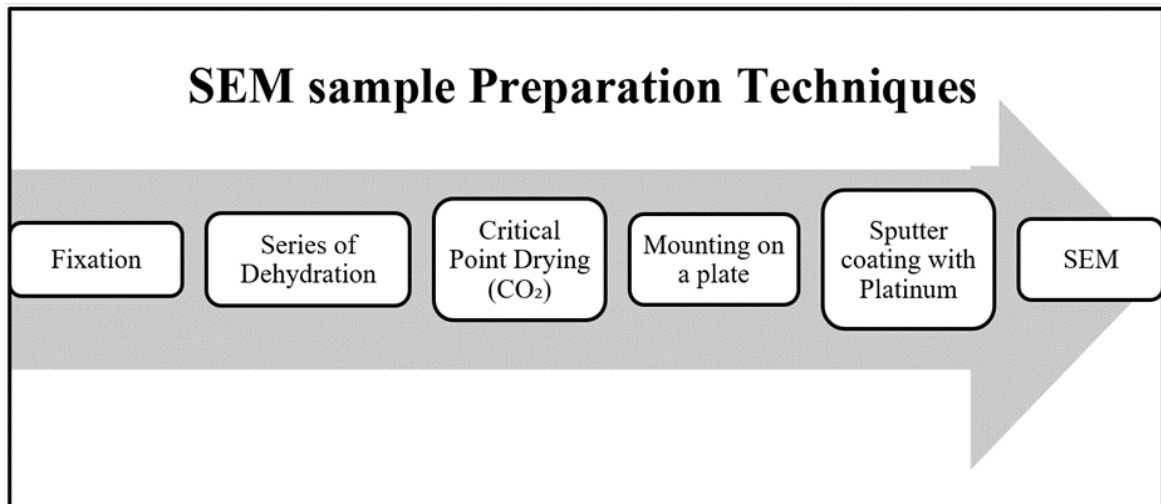


Figure 2: Flow chart for SEM sample preparation steps used in this study.

Table 1: Chemical solutions involved in Fixation and Series of Dehydration.

Step	Chemicals	Duration
Fixation	Formalin	1 week
Series of Dehydration	70% EtOH	1 hour
	80% EtOH	1 hour
	90% EtOH	1 hour
	100% EtOH	1 hour
	100% EtOH	1 hour
	100% EtOH	1 hour

Table 2: List of catalogue number for the polychaete specimen in this study.

Species	Catalogue no.	
	Holotype	Paratypes
<i>Buntalepis sarawakensis</i> sp. nov.	ASD 04258	ASD 04259 ASD 04260 ASD 04261 ASD 04262 ASD 04263
<i>Glycera tridactyla</i>		ASD 04264 ASD 04265
<i>Glycera alba</i>		ASD 04266 ASD 04267
<i>Glycera tessellata</i>		ASD 04268 ASD 04269
<i>Micronephthys sphaerocirrata</i>		ASD 04270 ASD 04271
<i>Onuphis eremita</i>		ASD 04272 ASD 04273
<i>Onuphis</i> sp. 1		ASD 04274 ASD 04275
<i>Onuphis</i> sp. 2		ASD 04276 ASD 04277
<i>Euchone</i> sp. 1		ASD 04278 ASD 04279
<i>Scoelelepis</i> (<i>Scoelelepis</i>) <i>squamata</i>		ASD 04280

Appendix C: Phylogenetic Analysis

Table 3: DNeasy Blood and Tissue Kit content and its volume provided.

DNeasy Blood & Tissue Kit	50 reps
DNeasy Mini Spin Columns in 2 ml Collection Tubes	50
Collection Tubes (2 ml)	100
Buffer ATL	14 ml
Buffer AL*	12 ml
Buffer AW1 (concentrate)	19 ml
Buffer AW2 (concentrate)	13 ml
Proteinase K	1.25 ml

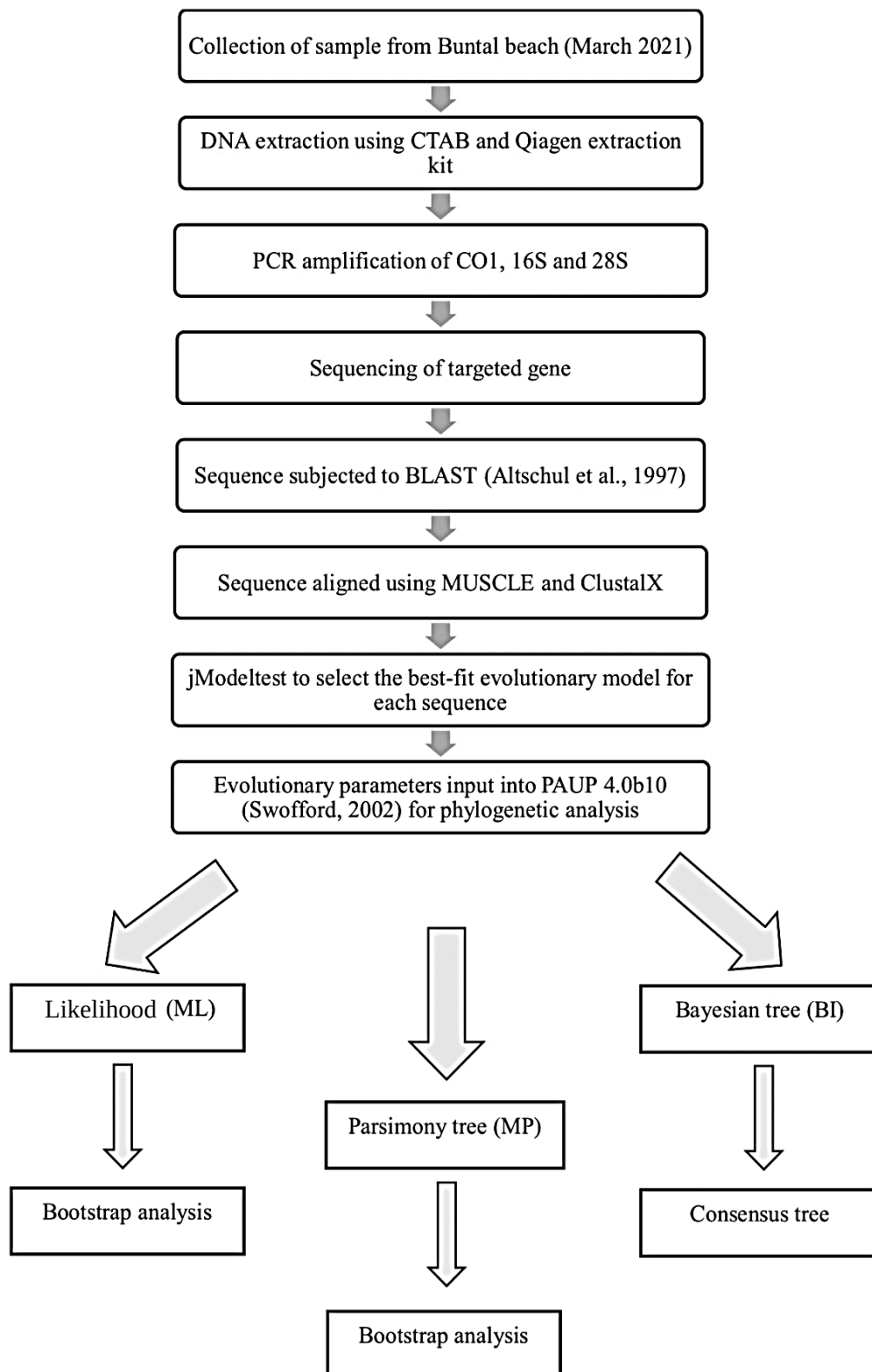


Figure 3: Flowchart showing the workflow of collecting, extracting and inferring phylogenetic analysis of *Buntalepis sarawakensis* sp. nov. using PCR sequencing of CO1, 16S mitochondrial region and 28S nuclear rRNA region