MORPHOLOGICAL AND PHYLOGENETIC ANALYSIS OF *CALIGUS SPP* ISOLATED FROM *LATES CALCARIFER* CULTURED IN FLOATING NET CAGES IN MALAYSIA

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FACULTY OF SCIENCE
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ABSTRACT

Asian seabass, *Lates calcarifer* (Bloch 1790) is the most marketable cultured fin fish in Malaysia. To date, cultured seabass is known to be parasitized by two species of *Caligus* namely, *Caligus epidemicus* and *Caligus punctatus*. However, the documentation regarding on their parasitism is still limited. Thus, the attention of this study is to investigate the occurrence of *Caligus* spp on *L. calcarifer* cultured in brackish water floating net cage, with the comparison between both monoculture and polyculture practices. The specific aims are to; (1) calculate the abundance of infection, (2) phenotypic characterization using scanning electron microscopy and (3) phylogenetic analysis based on 28S rRNA gene. *Caligus epidemicus* samples were isolated from all locations, comprises with more than 90% of the total isolates with the abundance as high as 2.236. It was followed by 47 individuals of *C. rotundigenitalis* (Yü 1933) and one *C. chiastos* (Lin and Ho 2003) which was isolated from polyculture practices with the abundance unit of 0.144 and 0.003 respectively. *C. punctatus* sample was nowhere to be found. SEM proved to be a useful identification tool whereby, clear phenotypic characteristic and anatomical body structure of each species precisely visualized. The images allowed the ordinal classification of the isolates to be re-examined. Molecular-based analysis via 28S rRNA region was carried out using; (1) neighbour-joining (NJ); (2) maximum parsimony (MP) and; (3) maximum likelihood (ML). The information gathered from the partial sequences of 28S rRNA gene revealed monophyletic relationships within the family *Caligus* in NJ and ML. In contrast, the MP analysis revealed *C. epidemicus* as a discrete monophyletic group within Caligidae. The interrelationships between the Caligidae lineages were supported as *Lepeophtheirus* forms a sister group relationship with *Caligus*. This *Caligus-Lepeophtheirus* group is well distinguished from other siphonostomatoids. Calanoida forms a basal group to the lineages. As a conclusion, *C. epidemicus* was the only consistent species to be found

infesting cultured seabass in both culturing systems within a wide range of water salinity. Despite the low numbers of *C. rotundigenitalis* and *C. chiastos* infestations, they may produce a serious challenge for *L. calcarifer* polyculture farming activity due to their capability of changing host particularly at 24 to 28 ppt. The morphology analysis confirmed that the isolated specimens were *C. epidemicus, C. rotundigenitalis and C. chiastos*. Molecular technique based on partial sequencing of 28S rRNA gene differentiates our isolates from other Copepoda taxa retrieved from GenBank™.

ABSTRAK

Asian seabass*, Lates calcarifer* (Bloch 1790) adalah ikan ternakan yang paling banyak dipasarkan di Malaysia. Sehingga kini, ternakan seabass diketahui dijangkiti oleh dua spesis *Caligus*; *Caligus epidemicus* dan *Caligus punctatus*. Secara keseluruhannya, dokumentasi berkaitan dengan parasit ini masih lagi terhad. Oleh yang demikian, kajian ini menjurus untuk mengkaji kewujudan *Caligus* spp ke atas *L. calcarifer* yang diternak di dalam sistem ternakan sangkar terapung air payau, dengan perbandingan di antara kaedah monokultur dan polikultur. Objektif spesifik adalah; (1) mengira kelimpahan jangkitan, (2) perbezaan fenotip dengan menggunakan teknik mikroskop elektron dan (3) analisis filogenetik berdasarkan gen 28S rRNA. *Caligus epidemicus* telah dipencilkan di setiap lokasi, merangkumi lebih 90% daripada jumlah keseluruhan pemencilan dan kelimpahan sebanyak 2.236. Ianya diikuti oleh 47 *C. rotundigenitalis* (Yü 1933) dan satu *C. chiastos* (Lin dan Ho 2003) yang dipencilkan daripada sangkar terapung polikultur dengan kelimpahan sebanyak 0.144 dan 0.003. *C. punctatus* tidak berjaya untuk ditemui. SEM terbukti sebagai satu teknik yang berkesan di mana, gambaran jelas kriteria fenotip dapat dilihat dengan struktur badan sebenar bagi setiap spesis. Gambaran sebegini membolehkan kajian untuk melakukan pengkelasan terhadap spesis yang dipencilkan dengan tepat dan jitu. Analisis molekular berdasarkan gen 28S rRNA dijalankan dengan menggunakan; (1) “neighbour-joining” (NJ), (2) “maximum parsimony” (MP) dan (3) “maximum likelihood” (ML). Maklumat yang terdapat daripada jujukan separa lengkap gen 28S rRNA menunjukkan hubungan ‘monophyletic’ di antara famili *Caligus* di dalam NJ dan ML. Sebaliknya, analisis MP merangkumkan bahawa *C. epidemicus* sebagai kumpulan yang berasingan di dalam Caligidae. Hubungan di antara Caligidae dapat dibuktikan dengan jelas dimana *Lepeophtheirus* adalah ‘sister group’ kepada *Caligus*. Hubungan *Caligus*-*Lepeophtheirus* ini dapat dibezakan daripada siphonostomatoids yang lain. Calanoida adalah dasar bagi

hubungan filogenetik ini. Sebagai rumusan, *C. epidemicus* adalah spesis yang sentiasa menjangkiti ternakan seabass di dalam kedua-dua sistem ternakan termasuk julat kemasinan yang luas. Disebalik jumlah pemencilan *C. rotundigenitalis* dan *C. chiastos* yang rendah, kedua-dua spesis ini boleh menyebabkan cabaran yang serius kepada *L. calcarifer* yang diternak secara polikultur disebabkan oleh kebolehan untuk menukar perumah di tahap kemasinan antara 24 sehingga 28 ppt. Analisis morfologi membuktikan dengan jelas bahawa spesis yang dipencilkan di dalam kajian ini adalah *C. epidemicus*, *C. rotundigenitalis* dan *C. chiastos*. Teknik molecular berdasarkan jujukan separa lengkap gen 28S rRNA membezakan ketiga-tiga spesis tersebut daripada kumpulan Copepoda yang diambil daripada GenBank™.

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LIST OF ABBREVIATIONS

bp	base pair
BLAST	Basic Local Alignment Search Tool
°C	degree Celsius
DNA	deoxyribonucleic acid
dNTPs	deoxyribonuleoside triphosphate
ML	Maximum likelihood
MP	Maximum parsimony
mtDNA	mitochondrial DNA
MEGA	Molecular Evolutionary Genetic Analysis
NJ	Neighbour-joining
ppt	part per thousand
PCR	Polymerase Chain Reaction
PAUP	Phylogenetic Analysis Using Parsimony
pmol	picomole
rRNA	ribosomal RNA

CHAPTER 1

Introduction

Aquaculture activities are facing severe challenges due to the parasitism of copepods (Ho, 2000). This type of parasite is known for their negative impact towards the growth, fecundity and also survival of their host (Boxaspen, 2006; Johnson *et al*., 2004). One of the most commonly reported is caligids. There are 90 species caligids from the genus of *Caligus* and 33 species of genus *Lepeophtheirus* so far reported from cultured and wild marine fish (Ho and Lin, 2004). Particularly in Asia, ten species which are considered as pathogenic species on fish farms and three of the species were reported to occur in Malaysia.

Based on previous records, the species of *Caligus* known to parasitize *L. calcarifer* has increase in time. In the year of 1985, there was no report of *Caligus* spp isolated during the survey on healthy and diseased seabass in Penang (Leong and Wong, 1990). However, in a survey on 2003 performed in Penang, reveals the fundamental discoveries of *Caligus epidemicus* Hewitt (1971) on *L. calcarifer*. In the same publications, another species; *C. punctatus* was found parasitizing *L. calcarifer*.

However, the documented infections were localized in Northern part of Malaysia, specifically in Penang and Langkawi. Moreover, the finding was almost solely reporting on their morphological descriptions and some quantitative descriptions on the parasite populations. So far, there is no published literature regarding molecular study on the parasitic genera in South East Asia. As contrast, these modernized approaches were

normally seen on the species of Caligidae which infested the salmonid culturing industry which outside South East Asia. Thus, little information is available regarding their molecular background and phylogenetic inferences. The discoveries were mainly focus on mitochondrial (mtDNA) and ribosomal RNA-encoded genes (rRNA). These targeted genes were utilized to infer the phylogenetic lineage within the Copepoda and as well as in Crustaceans (Braga *et al*., 1999; Jarman *et al*., 2002; Hurwood *et al*., 2003; Bucklin *et al*., 2003).

1.1 Justification of Studies

In general, the documentations relating to the parasitism of *Caligus* Müller (1785) in our native environment has been focus only at identification level. To summarize, it is still very far from complete. Therefore, further studies are deeply required in order to figure for high degree of understanding in order to combat against this parasite. As stated, a significant rise was observed in the number of species infecting cultured *L. calcarifer*.

Thus, this present study shall be able to provide with the information regarding the biodiversity of *Caligus* spp in four different states in Malaysia, namely, Johor, Kelantan, Penang, Sabah and Sarawak. The information regarding the abundances of infections from each site is very critical in determining which species is more dominant on the cultured *L. calcarifer*. Since, the primary stage in the development of parasitological study is the discovery and description of the parasites itself (Robert and Janovy, 2009). This study would therefore provide a comprehensive and detailed morphological structure of the isolated Malaysian species via Scanning Electron Microscopy (SEM). It involves

evaluating the specific phenotypic criteria to classify the genus, family and order grouping of the parasite.

Today, new modernized approaches have proven to be exceedingly powerful tools for resolving taxonomic problems. For example, the 18S and 28S rRNA sequences are able to generate phylogenetic signals which differentiate between each grouping up to species level. Therefore, the present study attempts to elucidate the the phylogenetic relationship of isolated species among the Copepoda. This would therefore be the pioneer information in exploring the genetic diversification between the species and other Copepoda in Malaysia.

By combining both phenotypic and genotypic approaches together with the quantitative descriptors, a concrete evaluation is therefore available for confirmation. Overall, the present study offers new information for the biodiversity of *Caligus* Müller (1785) in our native environment. This would include numerical diversification in terms of the abundance of the ectoparasite, diversification across several states in Malaysia and also genealogical diversity of isolates among the Copepoda.

1.2 Objectives of study

Specific aims were to:

1. To identify the species within genus *Caligus* based on morphological analysis.
2. To investigate the abundances of infection of *Caligus* on *L. calcarifer* in Malaysia.
3. To infer phylogenetic relationship of *Caligus* using 28S ribosomal RNA sequence.

1.3 Thesis organization

This thesis is comprised of seven chapters. Chapter 1 discussed more on the general background, objectives and the justification of present study. It was been further elaborated in Chapter 2 with the addition of relevant literatures. All of the objectives are specifically discussed in three separate chapters; Chapters 3 to 5. The next chapter (Chapter 6) summarize all of the findings. The final chapter (Chapter 7) concludes the output of the study. The following are the related papers that have been published or submitted for publication:

1. Kua, B.C.**,** Muhd. Faizul, H.A.H. (2010). SEM of three species of *Caligus* (Copepoda: Caligidae) parasitism on cultured marine fish at Bukit Tambun, Penang. *Malaysian Journal of Microscopy* 6 (9-13).
2. Muhd-Faizul H.A.H., Kua B.C., Leaw Y.Y. (2011). Caligidae infestation in Asian seabass, *Lates calcarifer*, Bloch 1790 cultured at different salinity in Malaysia. *Veterinary Parasitology* (in press).
3. Muhd-Faizul H.A.H., Subha, B., Kua B.C (2011). *Caligus* Müller, 1785 parasitizing cultured *Lates calcarifer* Bloch, 1790 at floating net cages in Malaysia. (Book of Abstracts of 8th Symposium on Diseases in Asian Aquaculture).
4. Muhd-Faizul H.A.H., Subha, B., Kua B.C (2011). *Caligus* Müller, 1785 parasitizing cultured *Lates calcarifer* Bloch, 1790 at floating net cages in Malaysia. (Manuscript submitted to *Journal of Aquaculture*).

CHAPTER 2

Literature Review

2.1 *Lates calcarifer,* Bloch 1790

2.1.1 Background

Historically, this fish was first described in 1790 by Marcus Elieser Bloch. The specimen was collected by Dutch merchants whom returning from the Indo-Pacific region (Grey, 1986). Nowadays, this species is highly regarded as one of the most important commercially cultured finfish within the region (Cheong, 1989). Basically, the adult is carnivorous, where feeds on fishes and small crustaceans such as prawns, while the juvenile only feeds on zooplankton. This species also widely known for their euryhaline and catadromous characters. It is localized in coastal, estuaries and also fresh water habitats within the tropical and semi-tropical regions of indo-west Pacific (Blaber, 2002). As a result, it is recognized with several local names such as barramundi in Australia, giant sea perch and anama in Papua New Guinea, sea bass and bhekti in India, sea bass in Thailand and Philippines, akema in Japan and Kakap in Indonesia (Dunstan, 1962; Rabanal and Soesanto, 1982). In Malaysia, it is commonly known as siakap or selunsung. In South East Asia, *L. calcarifer* is cultured in fresh, brackish and marine water systems, either in earthen ponds or cages (Kungvankij *et al.*, 1984). These types of systems are also well adopted throughout Malaysia (DOF, 2010). Highly delicious taste, fast growth rate and large size are among the dominant factors for their popularity in aquaculture (Cheong, 1989). Moreover, they are able to tolerate crowding, wide physiological tolerances and suitable for captive breeding and nursery.

2.1.2 Status of cultured seabass in Malaysia

Fish supplies in Malaysia consist of two important components, namely, capture fisheries and aquaculture. The capture sector is the major contributor to total fish production as compared to the involvement of aquaculture (DOF, 2010). However, on the emphasis of seabass productions, the contribution of aquaculture is becoming more evident (Figure 2.1). From the past two decades, the capture sector contributes to less than 2000 tonnes of seabass per year.

As stated, the production of cultured seabass in Malaysia may consist of brackish water ponds, brackish water floating net cages and also marine tank. It is known that the cage culture system is more popular among the rest (Figure 2.2). This culture technique is already established since 1970 by the Fisheries Department of Malaysia (Awang, 1986). However, the brackish water pond industry has started to increase their production within the last two years (Figure 2.2). The estimated marine tank production is only 8.40 in 1998 and increased to 34.08 tonnes in 2009 (DOF, 2010). Basically, the juveniles are cultured to harvestable size; 350 g to 3 kg, within six months to two years (FAO, 2008). The period largely depends on many factors, such as, environmental, disease, stocking density and nutrients.

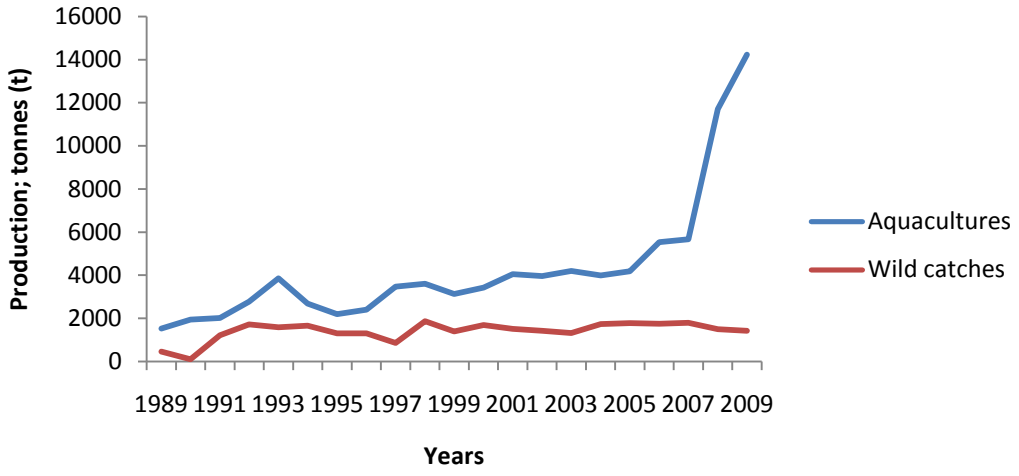

Figure 2.1: The estimated production of Asian seabass from aquaculture and wild from 1989 to 2009. Data were retrieved from the Department of Fisheries, Malaysia (DOF, 2010).

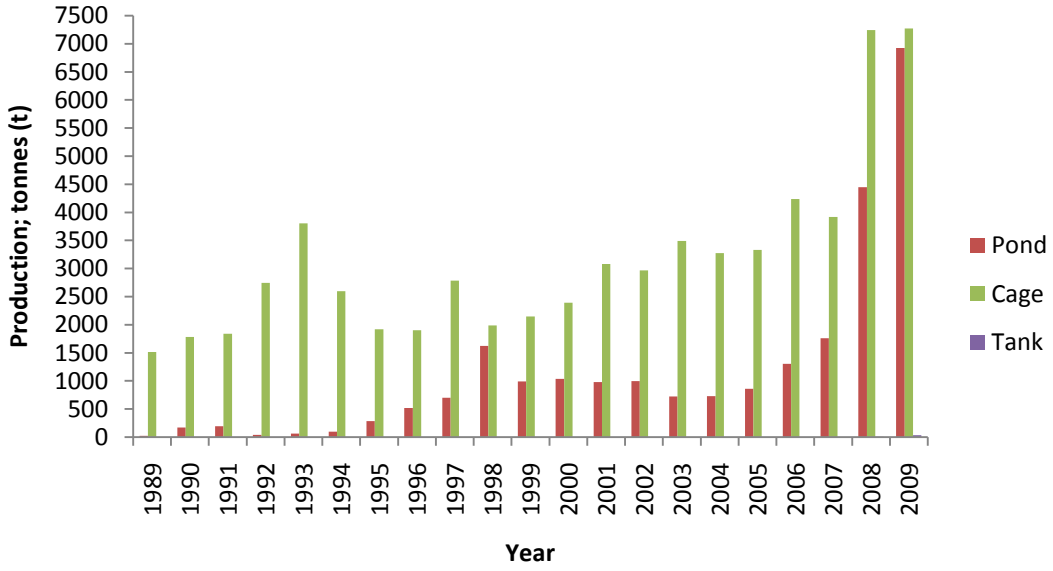

Figure 2.2: The estimated production of Asian seabass from floating cages, ponds and tanks from 1989 to 2009. Data were retrieved from the Department of Fisheries, Malaysia (DOF, 2010).

2.1.3 Diseases

The most limiting barrier for aquaculture is definitely disease (Kabata, 1985). The causative pathogens for spreading diseases are including parasitic organisms, bacteria, fungal and viruses. With regards to Asian seabass, numerous diseases have been reported to occur in Malaysia, for example, Vibriosis, Streptococcusis, Viral Nervous Necrosis, *Cryptocaryon irritants* and *Caligus* spp (Ransangan and Mustafa, 2009; Venmathi Maran *et al*.,, 2009; Lokanathan *et al*., 2010; Ransangan and Manin, 2010). In addition, malnutrition and environmental stresses such as temperature, low dissolved oxygen and poor handling may have the significant impact of disease on the cultured fish (Chonchuenbob, 1986; Harper and Wolf, 2009).

2.2 *Caligus* Müller, 1785

2.2.1 Background

Caligus Müller (1785) is a genus of ectoparasite family Caligidae (Subclass Copepoda: Order Siphonostomatoida). It is commonly known as sea louse due to its small size (Kabata, 1985). On the whole, this genus is regarded as the largest group among the Copepoda, whereby accounting for more than 200 species (Ho *et al*., 2000; Vo *et al*., 2008; Boxshall and El-Rashidy, 2009). This parasitic copepodid is among the most commonly reported to occur on marine and brackish water cultured fish throughout the world. Based on morphological structures, *Caligus* Müller (1785) is an arthropod due to the presence of an external skeleton, typical segmentations and jointed limbs (Kearn, 2004; Robert and Janovy, 2009). The scientific classification of this louse is as below.

Kingdom : Animalia

Phylum : Arthropoda

Subphylum : Crustacea Brünnich, 1772

Class : Maxillopoda Dahl, 1956

Subclass : Copepoda Milne-Edward, 1840

Order : Siphonostomatoida Thorell, 1859

Family : Caligidae Burmeister, 1834

Genus : *Caligus* Müller, 1785

2.2.2 Biology

2.2.2.1 Geographical distribution

The geographical distributions of *Caligus* Müller (1785) are widespread. Their tolerance toward water salinity, temperature and biochemical compounds largely differ among species. This type of distribution is best to observe on the salmonid culturing industry since it is the most documented area. As known, this extensive industry is very popular in Atlantic and Pacific Ocean of the Northern hemisphere. However, within the Atlantic waters, *C. elongatus* is known to threaten the industry, whereas in the Pacific, *C. clemensi* is the main threat. This type of distribution is also been observed in the Southern hemisphere, whereby *C. teres* and *C. rogercressyi* are known to parasitize the industry.

Among species, *C. elongatus* Von Nordmann (1832) and *C. clemensi* are the major species reported (Johnson *et al*., 2004). These two are known for their capability to create problem to the salmonid culturing industry in a few countries within the American, European and Asian continents (Johnson *et al*., 2004). There is a lot of literature documented on their identification, biology and also control measures. Within the Asian continent, there are seven species of *Caligus* Müller (1785) considered as killer species towards non-salmonid cultured fishes (Table 2.1). Among the listed species, *C. epidemicus* is known to be the most harmful species to its parasitized host (Venmathi Maran *et al*., 2009). Besides that, it is known to infect a wide range of hosts.

In Malaysia, there are eleven species known to parasitize both wild and cultured fishes (Appendix 1). Three of them are among listed killer species. However, these initial documentations on cultured fishes were only focused on floating net cages located in Penang and Langkawi. No other isolation has been documented from other culturing areas, systems and practices.

2.2.2.2 Environment and Habitat

Caligus Müller (1785) is commonly reported to be present in marine and brackish water around the world (Johnson *et al*., 2004). So far, only one species is known to live in freshwater within the Palaearctic ecozone; *C. lacustris* Steenstrup and Lütken (1861) (Kabata, 1992). In Malaysia, the same scenario is observed as all of the isolated species are originated from either brackish or marine water. Prior to this, it is hypothesized that the ancestral copepods is actually a marine epibenthic organism (Hairston and Bohonak, 1998). Some members of Copepoda such as Platycopoida, Mishoprioida, Harpacticoida and Cyclopoida are known to tolerate this type of environment.

However, it is known that *Caligus* spp is a photo-positive organism (Rohde, 2005). This assumption is based on the research on *L. salmonis* whereby, it is found that the swimming activity of naupli, copepodid and adult stages are all positively correlated with light intensity (Flamarique *et al*., 2000). Moreover, it is hypothesized that the feeding behaviour of nauplius larvae may be either planktotrophic or lecithotrophic (Rohde, 2005). The planktons need sunlight for photosynthesis, and the nauplius needs planktons to

survive. Thus, the *Caligus* spp is believed to live within the areas which are exposed to sufficient light and planktonic organisms.

Since the caligids is part of the sea ecology, the fluctuation of sea temperature also signifies the occurrences of caligids (Morton *et al*., 2004). It is believed that the life cycle of sea louse is strongly dependent on temperature (Castello, 2006). Basically, it is very typical for all crustaceans to live longer and grow larger at colder temperatures (Pike and Wardsworth, 2000). Thus, the increasing in water temperature will figure for shortened cycle, therefore the abundances of this parasite are more evident. This temperature-dependent life cycle has important implications for louse control on farms. They are likely to affect the geographical distribution of sea lice, potentially bringing new species into new areas.

2.2.2.3 Life cycle

The life cycle of caligids may consist of five major phases; naupli, copepods, chalimus, pre-adult and adult (Kabata, 1972). In between these phases, it is believed that there are ten moulting stages. However, this assumption is best suited for the life cycle of *Lepeophtheirus* spp rather than *Caligus*. This is because *Caligus* itself may consist of 8 to 11 moulting stages.

The cycle starts with mating process. It involves the transfer of spermatophores of an adult male into the genital opening of either pre-adult or adult female (Ritchie *et al*., 1993; Kearn 2004). During this process, the mating pair holds in a unique position known

as pre-copula for several hours (Kearn, 2004) (Figure 2.3A). The male releases two spermatophores and implants them on the female genital segment by using its second thoracopod. Finally, each spermatophore developes into two egg string within a thin tube called ovisaic (Figure 2.3B).

After maturing, these eggs hatch into the first phase known as free swimming nauplius larvae (Figure 2.3C). These larvae are relatively very small, un-segmented body and equipped with only three limbs (Rohde, 2005). These larvae are planktotrophic or lecithotrophic and do not attach to any host (Lewis, 1963; Rohde, 2005). Moreover, it has been discovered that nauplius larvae respond towards light exposure. They actively swimming upwards and followed by passive-sinking in the presences of light. This phase later evolves into copepodid (Figure 2.3D).

Basically, the copepodid is actually an infective larval stage. At this point, the copepodid will attach to the host by using the second antennae. Once attached, it uses the maxillipeds to move onto the host surface to find a suitable site to settle (Shram, 1993). Thus, at this point it already established itself as a parasite. However, based on further study on the behaviour of copepodid of *L. pectoralis* and *L. salmonis*, it is believed that if it is attached to an unsuitable host, the copepodid is capable of resuming swimming (Bron *et al*., 1993).

Once again after the moulting process has taken place and the copepodid transforms into chalimus (Figure 2.3E). At this point, this ectoparasite shows a different characteristic from the previous nauplius & copepodid whereby it produces a chitinous frontal filament.

This filament is used for attachment and feeding apparatus (Jones *et al*., 1990; Bron *et al*., 1993). It is believed that at this phase, the energy usage is very minimal since it largely depends on the filament for attachment. Thus, it can be directed towards growth and development.

Then, the next phase is pre-adult. At this point, pre-adults are not attached by frontal filament anymore (Figure 2.3F). They are fully capable of free movement over the host body. Finally, it evolves into the adult phase.

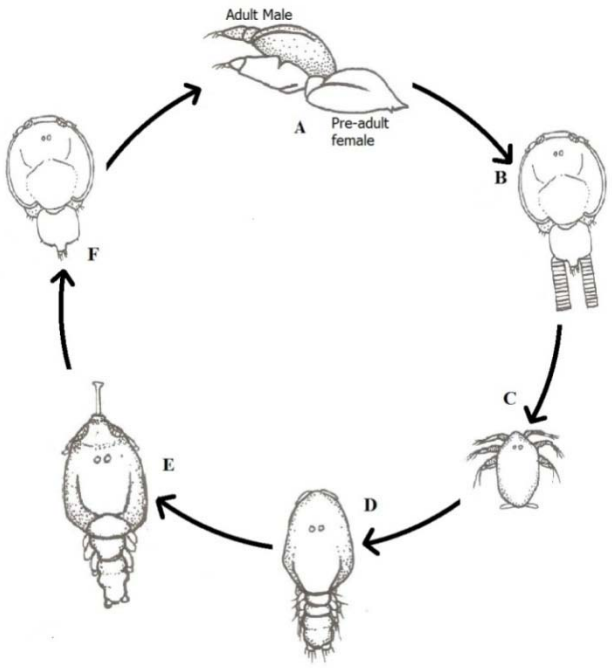

Figure 2.3: Basic life cycle of *Caligus* spp based on Kabata (1979) and the structure of *C. epidemicus* Hewitt (1971). (A) pre-copula position; (B) adult female with eggs; (C) naupli; (D) copepods; (E) chalimus; (F) adult female and male. Not all stages are included. Based on Kabata (1979) and Hewitt (1971).

2.2.2.4 Targeted Host

The caligid host range is one area of interests due to the diversification. Some species are highly regarded as host-specific and there are also species which are not. For example, *Salmon salar* industry is facing severe problem due to parasitism of *L. salmonis* and *C. elongatus.* However, *L. salmonis* is highly specific to this host, while *C. elongatus* is known to parasitize several different hosts. In Malaysia, all of the isolated species are known for their low-host specificity characteristic. For example, *C. epidemicus* is acknowledged to have a wide range of hosts. It includes *L. calcarifer*, other species of finfish and also *Peneaus monodon*.

The mechanism of infection starts with the first infective phase, which is the copepodid. At this phase, it is known that this ectoparasite is able to choose whether the infected host is suitable or not. So, it is assumed that at this level, the copepodid will randomly attach to any fish until it finds a suitable one to settle. This host finding mechanism has a significant relationship with light intensity. The suitable terms is called phototactic (Kearn, 2004). According to Flamarique *et al*. (2000), copepods are positively correlated with light intensity. The knowledge about the targeted host has proven useful for species identification. For example, Leong (1984) had misidentified *C. multispinosus* as *C. rotundigenitalis* which was isolated from *Pampus chinensis* (Euphrasen, 1788). It is acknowledged that *C. multispinosus* is highly specific to silver pomfret, and there is still no reported case corresponding to the latter species. Thus, it is concluded that the isolated species is *C. multispinosus*.

2.3 Impact of *Caligus* spp on aquaculture

2.3.1 Concern

The most serious issues about the parasitism of *Caligus* spp corresponds towards the mortality, health status of the cultured fishes and economic losses (Boxshall and Defaye, 1993; Pike and Wadworth, 1999). On the basis of disease, mortality may be directly caused by the development of infections, stress factor, osmoregulatory failure and respiratory impairment (Brandal and Egidius, 1979; Finstad *et al*., 2000). All of these factors are related with the infection of *Caligus* spp on the host. There are many literature which investigates these issues (Johnson *et al*., 2004).

The presences of the caligids on fishes are common. In small numbers, this ectoparasite may only cause stress to the infected host (Ho, 2000). However, under certain unfortunate circumstances, the population starts to explode and their abundance becomes more evident. At this level of infection, they are able to cause skin lesions and open wounds. For example, it is known that in most cases, the copepodid, chalimus, pre-adult and adult stages only cause minor host tissue responses (Roubal 1994; Pike and Wadworth, 1999; Johnson *et al*., 1996). However, in high abundance, these stages can definitely cause significant pathological effects that can cause mortality (Lin *et al*., 1994; Wu *et al*., 1997).

As a result, this problem causes a huge impact on the economy. For example, Malaysian cage culture industries have faced losses of USD 1.3 million and the majority have been related to the presence of parasites (Wong and Leong, 1987). Besides mortality, the infections may also result in decreasing value of the infected fishes. This is due to the

fact that the fish are injured and wounded. The losses faced by the salmonid culture industry have been widely discussed. Another matter of concern is the transmission of these diseases onto the wildlife populations. This situation could occur since the cultured area is located within the wild life populations. Although these populations are scattered over vast distances, the migration pattern of certain host species such as *L. calcarifer* lead to higher probability for them to be infected.

2.3.2 Attachment onto host

The attachment of this parasite onto a host is occurring at the copepodid, chalimus and adult phases. However, the mechanism and damages caused by these three phases differ from each other. Besides that, each species possesses its own site of preferences, either settle down onto the body, fins or gill cavity. During the initial attachment by copepodid, it is attached to the host by using the maxillipeds (Figure 2.4A). The first and second antennae (Figure 2.4A) are in a position close to the host. If it chooses to localize onto the host, it will hook the second antennae by repeated stabbing actions. Then, cement is secreted from their frontal gland to finalize the process. The damage on the host is derived by the stabbing actions.

At the chalimus stage, the attachment is triggered by the formation of the frontal filament (Figure 2.4B). The chitinous filament is directly inserted through the epidermis of the host from the frontal plate. However, it is known that this attachment appears to cause minimal disruption onto the host (Jones *et al*., 1990).

In an adult stage, the cephalothorax is the ideal apparatus for attachment at slippery surfaces and strong water currents (Kabata, 1979). The edge of the shield is sealed by a peripheral flap that acts as a marginal valve when suction is generated. It prevents the influx of water between the edges of the shield and the host, while, the posterior sinuses permit water to escape from beneath the cephalothorax.

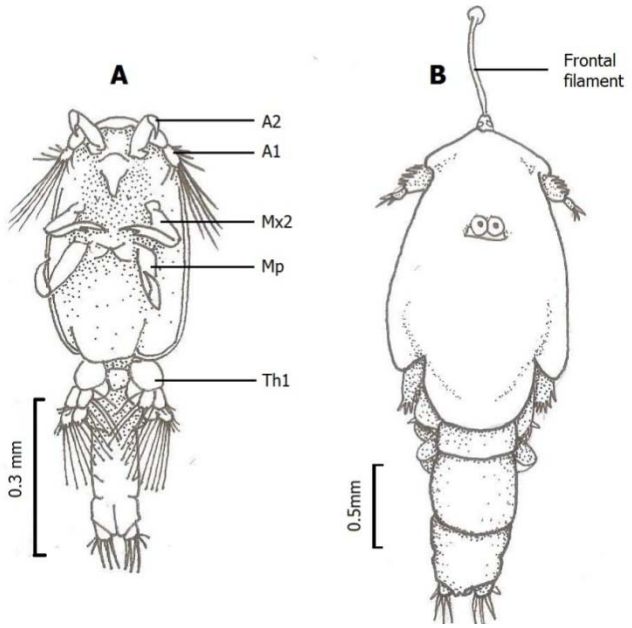

Figure 2.4: The attachment apparatus of *Caligus* spp. (A) Ventral view of copepods of *C. spinosus* redrawn from Kearn (2004) and (B) ventral view of chalimus of *C. rapax* redrawn from Robert and Janovy (2009).

2.3.3 Feeding activity

Primary disease symptom is developed as these parasites feed on host mucous, tissues and blood (Johnson *et al*., 2004). In the salmonid culture industry, the most damage is caused by their feeding activity. Erosion of the epidermis by the parasite will affect the water balance of fish, encouraging leakage of body fluids, with loss of proteins and electrolytes.

All caligids lineage shares the same feeding mechanism and apparatus whereby, it depends largely on their siphonostomatoid mouth cone (Figure 2.5A). This mouth cone lies at the midline of the cephalothorax. It is equipped with the upper lips (labrum), lower lips (labium) and a pair of mandible (Kabata, 1992). Kabata (1974) suggested that this mouth cone is folded back and pointing in a posterior direction along the ventral surface of the body when moving.

The mandible is the primary feeding appendage for all crustaceans (Figure 2.5B) (Robert and Janovy, 2009). In the Copepoda, the structure of the mandible is among the specific criteria in which differentiates between the orders. When feeding, the mandibles extend through the distal opening and scrape onto the epidermis of the host. Besides the mandibles, the cephalothorax is well-equipped with several feeding appendages such as the maxilla and maxilipeds (Robert and Janovy, 2009). These two hook-like appendages help to penetrate through the host epidermis.

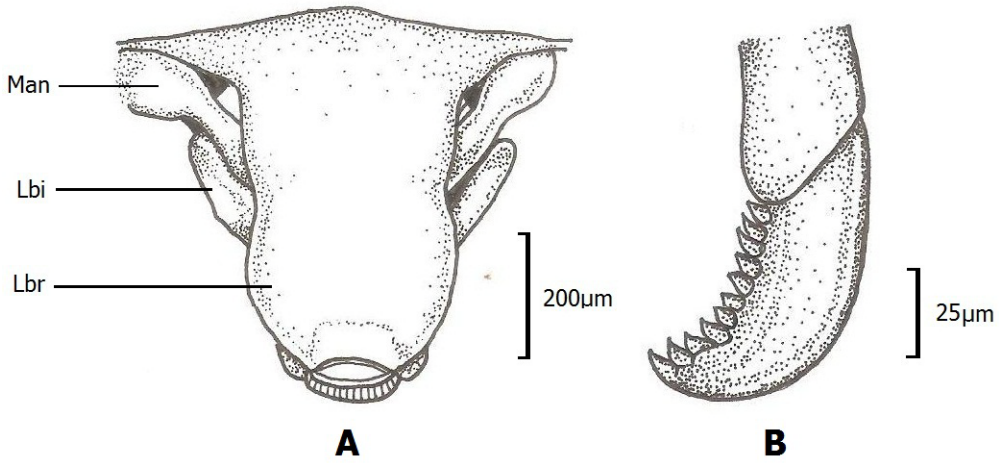

Figure 2.5: The mouth structure of *Caligus* spp. (A) mouth tube (siphon) with mandible redrawn from Kearn (2004) and (B) whole structure of the mandible redrawn from Robert and Janovy (2009).

2.3.4 Vector for secondary infection

Since their attachment and feeding activities results in significant mechanical injuries, it is assumed to be a vector for other pathogens. There are several breakthrough studies on *L. salmonis* which supports this assumption. It is known to be a vector for Infectious Salmon Anaemia (ISA), Infectious Pancreatic Necrosis and also furunculosis. However, so far there is no specific evidence that this caligids are involved in the transmission of other pathogenic organisms.

2.4 Morphology and molecular based identifications

2.4.1 Background

Principally, a character is a unique underlying feature or attribute possessed by an organism which can be used for comparison with other organisms. This may involve both phenotype and genotype characters. Overall, the characterizations of crustacean parasites primarily depends on phenotypic approaches rather than genotypic. To date, *L. salmonis* and *C. elongatus* are two most documented caligids. It covers both areas of characterization; morphology and molecular based identifications.

2.4.2 Morphology-based characterization

As a member of the Crustacea, *Caligus* spp. is characterized by their external skeleton, repetitive series of segments and jointed limbs (Kearn, 2004).The *Caligus* spp. is known to possess a podoplean-type body structure (Figure 2.6). This is where the division of prosome and urosome is located anterior to the fifth leg-bearing somite. This parasite is known to possess tubular buccal apparatus or known as siphon. This is the key attribute for all siphonostomatoids.

All caligid body consist of four tagmata, namely, cephalothorax, fourth leg bearing segment, genital complex and abdomen (Kearn, 2004). These morphological structures are very typical for its family and therefore, both *Lepeophtheirus* and *Caligus* are built on the same basis. However, each genus possesses its own unique characteristics. *Caligus* spp. differ from *Lepeophtheirus* spp by the presence of a pair of lunules at their frontal plates

(Figure 2.7B). In addition, the presence of their fourth thoracopod differentiates them from *PseudoCaligus* (Figure 2.7B).

The most characteristic feature of Caligidae is the structure of their sucker-like attachment organ or known as cephalothorax (Figure 2.7A and 2.7B) (Kearn, 2004; Rohde, 2005; Robert and Janovy, 2009). This suction disc is sealed by a frontal plate and marginal membranes. The posterior rim of the sucker is created by their third thoracopod (Figure 2.7B). The dorsal cephalothorax is smooth and roughly rounded, while the ventral structure is very different. It is equipped with feeding and attachment appendages. The fourth leg bearing segment is free, it does not attach to any appendages or others limbs (Figure 2.7A). It is located between the cephalothorax and genital segment. The structure of this fourth thoracopod varies between species. It consists of an endopod and exopod region, with the addition of several number of spines.

The next tagma is known as genital complex (Figure 2.7A). This complex plays an important role during the mating process (Kearn, 2004). It is attached to the posterior portion of the fourth leg bearing segment. The genital complex for each species has varying sizes and proportion. For the male, this complex is much smaller than the female. The final tagma is the abdomen (Figure 2.7A). It is a small organ attached posteriorly to the genital segment. There are two terminal uropod bearing setae posterior to the abdomen.

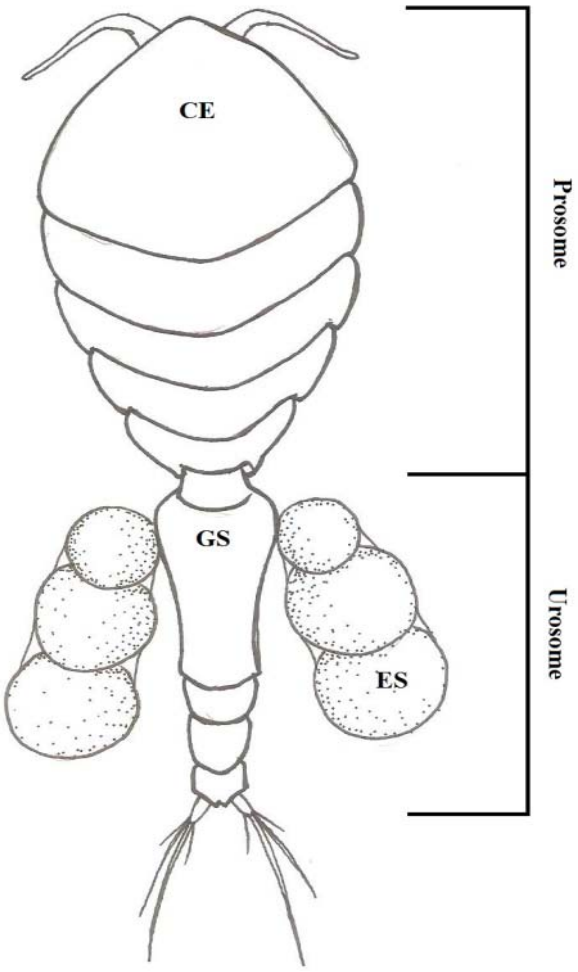

Figure 2.6: Basic Podoplean body plan of copepods. Abbreviations: CE, cephalothorax; CR, caudal rami; ES, egg sac; GS, genital segment; PR, prosome; UR, urosome. This picture is re-drawn from Rohde (2005).

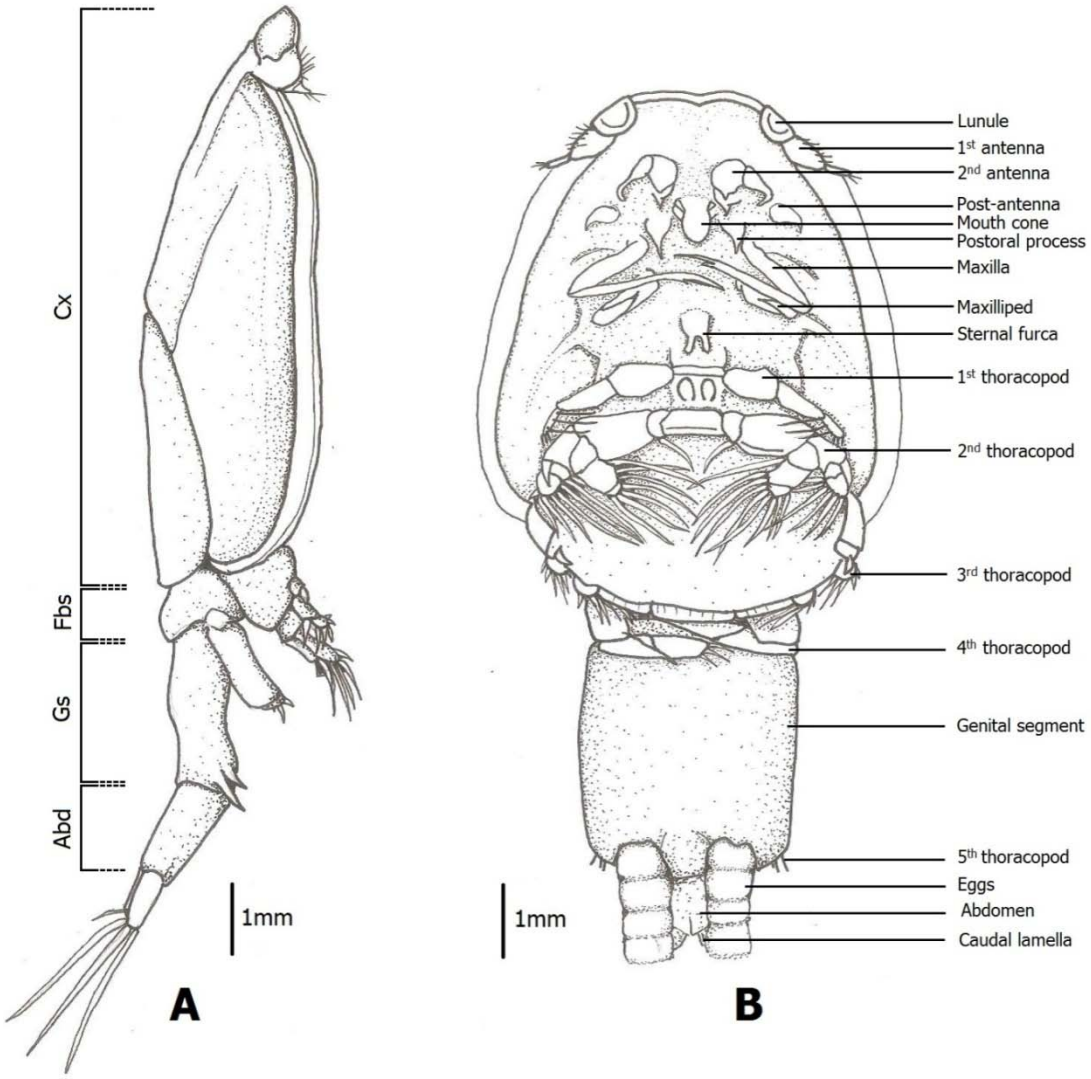

Figure 2.7: Illustrated figures of *Caligus curtus* Muller (1785) redrawn from Parker *et al*. (1968). (A) Lateral view of adult male and (B) Ventral view of an adult female. Abbreviations: Cx, cephalothorax; Fbs, fourth-leg bearing segment; Gs, genital segment; Abd, abdomen.

2.4.3 Molecular-based characterization

Characterizations based on molecular units and approaches are mainly inferred from the genetic information present in mitochondrial and ribosomal DNA (rDNA) (Papetti *et al*., 2002). The genes incorporated within the mitochondrial evolve at verifying rates (Zardoya and Meyer, 1996). It is assumed that mitochondrial genes are more suitable for population genetic studies since they accumulate substitutions up to 10 times faster than the nuclear genes (Shearer *et al*., 2002; Ballard and Whitlock, 2004). Besides that, mitochondrial genes are also more affected by genetic drift and fixation as compared with the nuclear DNA (Ballard and Whitlock, 2004). It is selected for inferring genetic structure, gene flow or phylogenetic relationships if the nuclear marker fails (Shoemaker *et al*., 2003).

2.4.3.1 Ribosomal RNA (rRNA) genes

Ribosomal RNA (rRNA) is an essential element for protein synthesis. Therefore, the presence of this gene is permanent in all living organism with the exception of viruses. It is acknowledged that rRNA is incorporated within the genome by multiple genes whereby the repetition varies up to several hundred in eukaryotes (Lewis, 1997). It is assembled within the genome as tandem repeats of a nuclear ribosomal gene cluster (rDNA) (Figure 2.8).

The arrangement of these repetitive segments starts with the non-transcribed spacer (NTS) region at the 5’ end. It is followed by the external transcribed spacer (ETS) and finally ends up with the rRNA genes (18S, 5.8S and 28S). However, the rRNAs are separated by two internal transcribed spacer; ITS-1 and ITS-2. Altogether, this arrangement

is critical for the transcription to occur. The spacers provide with all the processing signals in yielding a larger precursor molecule (Hillis and Dixon, 1991).

The ribosomal RNA genes are built by a core and expansion regions (Clark *et al*., 1984). The characteristic of these two regions are different from each other as the core region is highly conserved while the expansion regions are variable. Due to this factor, the core regions are able to differentiate organisms up to species level. The variability within the expansion regions are caused by repetitive motifs, insertions or an accumulation of single nucleotide polymorphism (Hillis and Dixon, 1991). Thus, due to the uniqueness, the genes have been utilized to infer molecular phylogenetic analyses, genetic population studies and systematic.

Conserved region of rDNA may consist of extremely precise information which is very specific for species identification. This is denoted by a unique nucleotide arrangement within the region. Each species possesses a specific base line-up. Analysis of RNA for taxonomic purposes focuses on the three rRNAs namely, 5.8S, 18S and 28S with the most commonly utilized is the 18S and 28S.

Out of all, the 18S rRNA gene is most commonly used in molecular characterization and phylogenetic studies. This is because the slow evolving characteristic of the sequences (Hillis and Dixon, 1991). On the other hand, the 28S rRNA is larger and the expansion regions are more variable (Hancock *et al*., 1988). Hence, the 28S rRNA has been used in population genetic studies, as well as molecular systematic studies (Babbitt and Patel, 2002; Stevens *et al*., 2002; Sawabe *et al*., 2003; Mallatt *et al*., 2004).

In addition, the spacers region has also been used for molecular phylogenetic and systematic purposes (Hillis and Dixon, 1991). Among the spacers, the NTS is the most rapidly evolving, while the transcribed spacers are more conserved (Hillis and Dixon, 1991). The NTS region is also known to be differ between individuals of the same species (Jorgensen and Cluster, 1988). Despite this, several population genetic studies have been performed using this region (Cunningham *et al*., 2003; Printzen *et al*., 2003; Gupta *et al*., 2004).

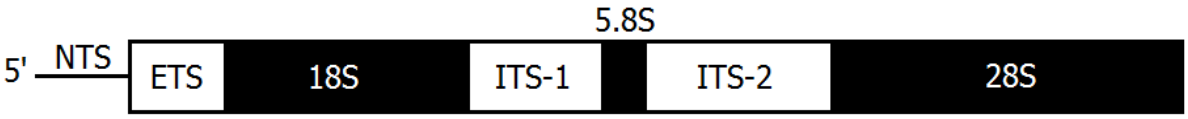

Figure 2.8: The arrangement of an eukaryotic rDNA tandem repeat.

2.5 Phylogenetic relationships of Copepoda

2.5.1 Background

The first establishment of Copepoda phylogeny was proposed by Kabata (1979). The study was based on the morphological descriptions of seven different orders, namely, Calanoida, Cyclopoida, Harpacticoida, Misophrioida, Monstrilloida, Poecilostomatoida and Siphonostomatoida (Figure 2.9). It is suggested that each order form a monophyletic clade. However, the total taxa split into two distinct groups, gymnoplea and podoplea. The gymnoplean group consist of only Calanoida, while the rest are incorporated into the podoplean group. Besides that, it was hypothesized that the Poecilostomatoida and Siphonostomatoida evolved from Monstrilloida.

The cladistic analysis proposed by Ho (1990) recognized an additional order, namely is the Platycopoida into the gymnoplean clade. Differently, Kabata (1979), hypothesized that the clade was the sister group to the remaining copepod orders. Within the main group, the Monstrilloida is placed closer to Cyclopoida. This branching pattern is dissimilar with the one proposed by Kabata (1979), whereby the Monstrilloida is among the most primitive taxon.

The copepod relationships were further revised by Huys and Boxshall (1991) (Figure 2.10). The study have been widely accepted and recognized. Similar with the first two, the Calanoida order branched out from the main group. However, in this phylogeny, Calanoida does not form in a monophyletic relationship with Platycopoida as proposed by Ho (1990). In fact, the Monstrilloida was found to incorporate into the main group rather

than accompanied by Cyclopoida. In fact, the cyclopoid is grouped together with Misophrioida and Gelyelloida which branched out from the main group constituting Poecilostomatoida and Siphonostomatoida.

As the fundamental discovery of reliable analytical techniques in molecular biology has already been established, Braga *et al*. (1999) focused on the information generated from the 28S rRNA. The studies were based on three families; Poecilostomatoida, Calanoida and Harpacticoida. The results showed that the relationships were very different from the morphology-based phylogeny. It was found that the Poecilostomatoida form the basal taxon to the monophyletic group constituting of both calanoids and harpacticoids.

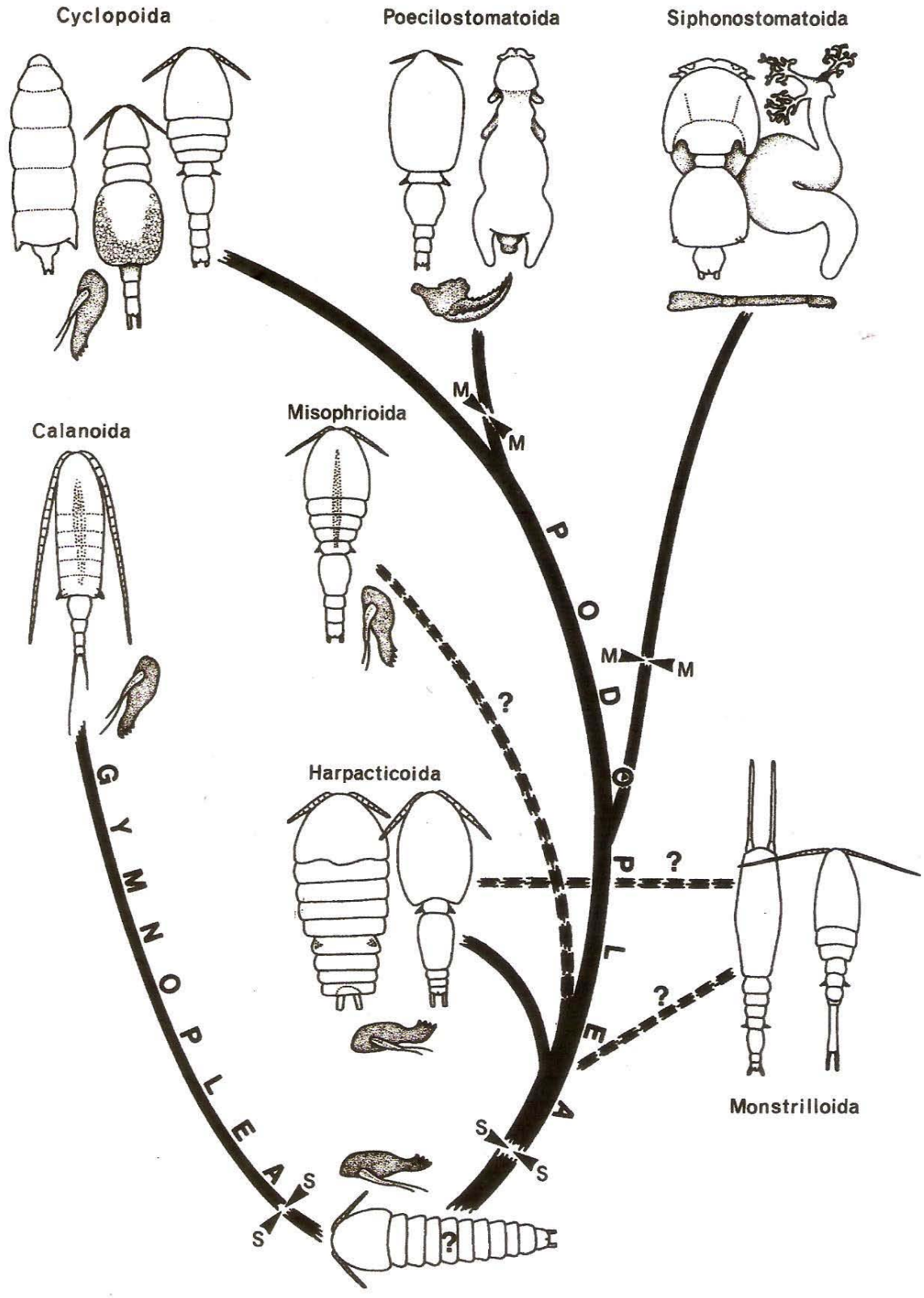

Figure 2.9: Copepoda relationships proposed by Kabata (1979).

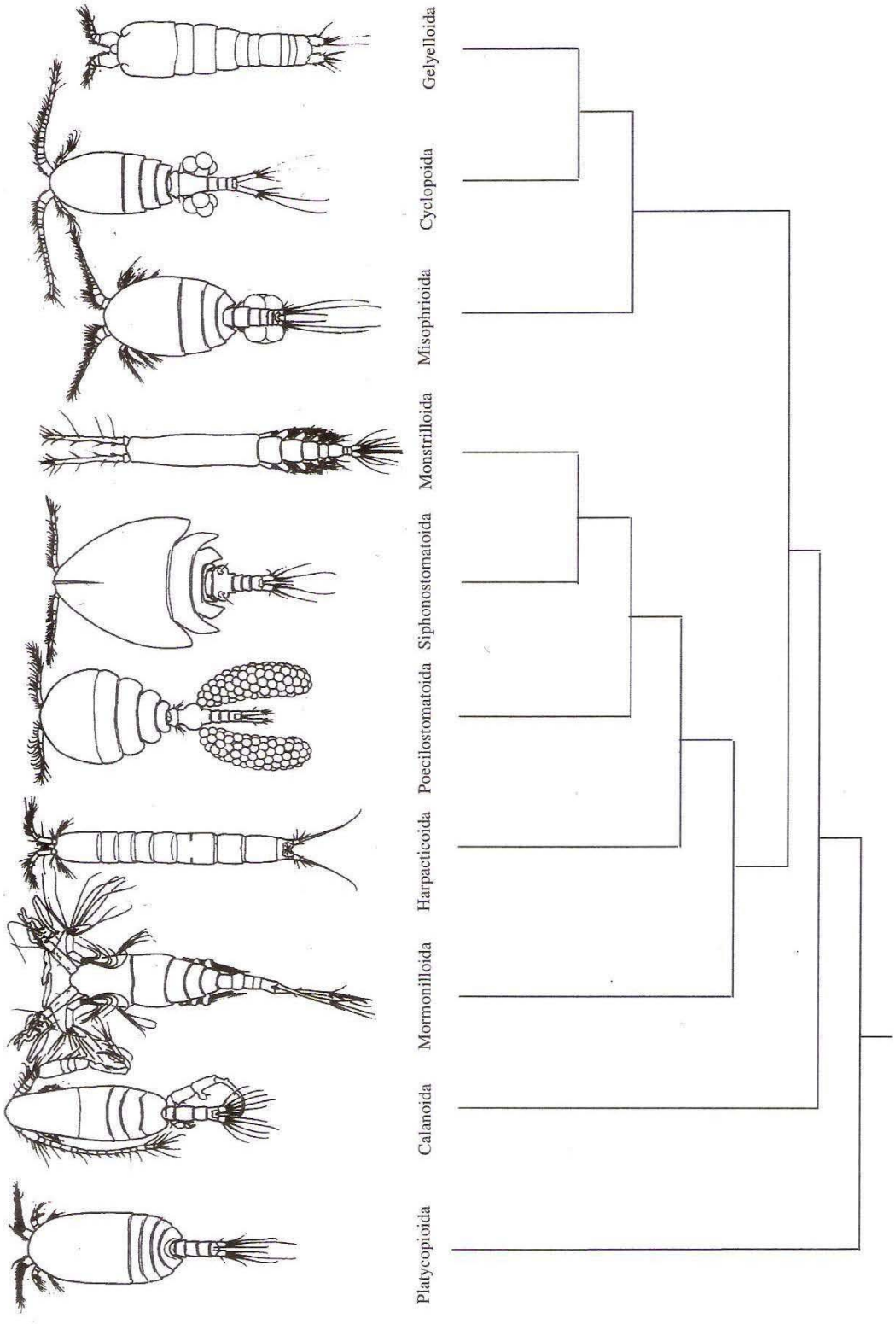

Figure 2.10: Copepoda relationships proposed by Huys and Boxshall (1991)

CHAPTER 3

Abundance of infections of *Caligus* spp. on Cultured Asian Seabass, *Lates calcarifer*, Bloch 1790 in Malaysia

[All the data from this chapter has been published in Veterinary Parasitology Journal and presented at the 3rd Symposium on Cage Aquaculture in Asia and 8th Symposium on Diseases in Asian Aquaculture]

3.1 Introduction

In Malaysia, *Lates calcarifer* is cultured in fresh water, brackish water and also marine systems, in either earthen ponds or cages (Kungvankij *et al*., 1986). There is high demand on this species as it can be produced throughout the year at a reasonable price. Thus, higher productivity is desired by increasing the stocking which might facilitate parasitic transmission (Kabata, 1985). It is reported that the Asian aquaculture sector including Malaysia are facing severe challenges due to parasitic copepod invasion. Such parasites are known to possess a single host-life cycle and reproduces rapidly. This characteristic is an advantage for them to alter and proliferate in aquaculture since the culturing area is condensed with their targeted host. Basically the information on these parasites is still rarely documented in Asian countries including Malaysia.

The first report on *Caligus* parasites on Malaysian fishes was in 1984 (Leong, 1984). There was a big gap in between that until Venmathi Maran *et al*., (2009) reported five species of *Caligus* that have been identified to cause problems to the aquaculture sector in Malaysia; *Caligus chiastos*, *Caligus epidemicus*, *Caligus rotundigenitalis* and *Caligus punctatus*. Of these, only *C. epidemicus* and *C. punctatus* infest *L. calcarifer*.

Kua and Faizul (2010) stated that *C. epidemicus*, *C. chiastos* and *C. rotundigenitalis* were commonly found in cultured marine fish at Bukit Tambun, Penang.

The previously documented locations were only centralized at the northern region of Malaysia; Langkawi and Penang. Larger sets of documented areas would likely support more concrete evaluations. There is one important question that has yet to be answered; *L. calcarifer* are more prone to how many species of *Caligus*, and how about other states in Malaysia? Thus, the present study was carried out to determine the occurrence and adaptability of caligid infection on seabass cultured at varying salinity in Malaysia. Besides, a comparison of this parasite invasive ability between culturing systems and practices was also carried out. This is vital as seabass is able to adapt to a wide range of water salinities.

3.2 Methodology

3.3.1 Sample collection

Seabass cultured from four states, namely Sarawak (Santubong and Semantan), Johor (Gelang Patah and Pendas), Penang (Bukit Tambun) and Kelantan (Semarahan) were obtained (Figure 3.1).

Figure 3.1: Location of selected sampling sites. Santubong; B, Sematan; C, Bkt. Tambun; D, Semarahan; E, Pendas; and F, Gelang Patah.

The fish samples were collected from two different brackish water culturing systems (earthen pond and floating cage) with monoculture and polyculture practices. Sample size ranging between 5 and 70 seabass were collected randomly using fish net at floating cages and fishing rod at pond. The randomly selected fishes were not restricted to certain a size, types of treatment applied by the farmers and their origin. The water salinity of each sampling site was determined using a multi-probe water quality instrument (YSI 556 MPS, USA). Four readings were taken at each site.

3.3.2 Collections of parasites

The fish was treated with fresh water for 15 minutes to collect the parasites and equipped with aeration supplies. After returning the fish to the cages, the water was examined for the presence of *Caligus*. The dislodged parasites were collected and counted in order to determine the prevalence, mean abundance and mean intensity. The parasites were preserved in 70% ethanol for further identification.

3.3.3 Identification

The parasites were stained using Alum– carmine for identification. The specimens were observed with a dissecting microscope (Leica Zoom 2000 and DM 5000) at eye piece magnifications of 50–400x. Identification was based on the morphological features according to Hewitt (1971), Lin and Ho (2003) and Venmathi Maran *et al*. (2009). Caligids were sorted and photographed. The prevalence, mean abundance and mean intensity were calculated based on Bush *et al*. (1997).

The related formulas are as below:

$$\text{Prevalence (\%)}: \quad \frac{\text{Number of infected host}}{\text{Total number of fish examined}} \times 100$$

$$\text{Mean abundance}: \quad \frac{\text{Total number of isolated parasite}}{\text{Total number of fish examined}}$$

$$\text{Mean intensity}: \quad \frac{\text{Total number of isolated parasite}}{\text{Total number of infected fish}}$$

Some samples were sent to Prof. Ju Shey Ho from Department of Biological Sciences, California State University, CA for confirmation in identification.

3.4 Results

Two different brackish water culturing systems (earthen pond and floating cage) with monoculture and polyculture practices were investigated for the occurrence of *Caligus* spp on *L. calcarifer*. Polyculture practices comprises mainly snapper (*Lutjanus* spp), grouper (*Ephinephelus* spp) and seabass (*L. calcarifer*), while monoculture only consists with Asian seabass. Each sampling site differs in salinity levels, ranging from 5 to 28 ppt (Table 3.1). A total of 326 *L. calcarifer* specimens were collected in this study. The sample size collected at each location was varied according to the stock available and cooperation of farmers. Mean size of collected fishes were summarized in Table 3.2.

777 specimens of caligids were isolated throughout the study, consisting of three different species; *C. chiastos*, C. *epidemicus* and *C. rotundigenitalis*. These parasitic organisms were normally attached onto the body of *L. calcarifer* (Figure 3.1). However, there were some specimens which attached to the operculum chamber. Among these, *C. epidemicus* was the only species consistently causing threat in all culturing areas, constituting for up to 93.8% of the total isolate (Table 3.2). Its presence was recorded at all locations with different salinity level; from 5 to 28 ppt. The numbers of the other four species were relatively very small; 45 for *C. rotundigenitalis* and only one *C. chiastos* specimens. The isolations of these two species were restricted only to polyculture floating cages. Their presence were observed within a narrow salinity range; 25 to 28 ppt.

The total mean abundance of *C. epidemicus* was the highest, with 2.236, followed by *C. rotundigenitalis*, 0.144 and *C. chiastos* with only 0.003. This abundance pattern was observed throughout the sampling whereby *C. epidemicus* accounting for the highest (Table 3.2).

The species identifications were primarily based on the morphological structures of the isolates observed under a compound microscope (Figure 3.2). The specimens were sorted and grouped together based on the similarity of their basic structures. The morphological differences between the isolated species were discussed thoroughly in the next chapter. However, a further clarification by an expert was required since the compound microscope allows viewing with eye piece magnification of 40-500X. Some of the samples were sent to Prof. Ju Shey Ho from Department of Biological Sciences, California State University, CA for confirmation in identification.

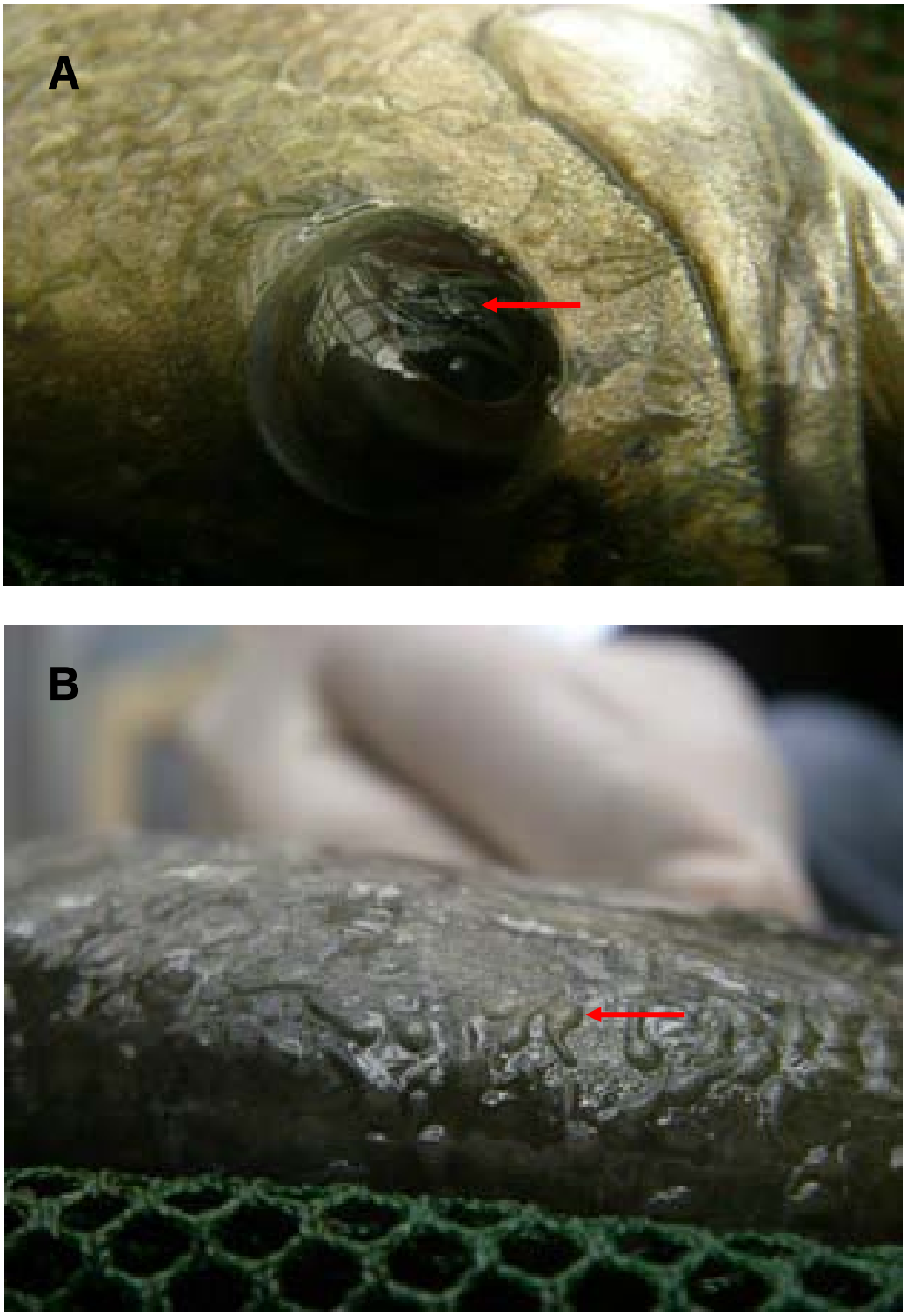

Figure 3.2: An Asian seabass, *L. calcarifer* infected with *Caligus* spp. A: attached to eye and; B: body.

Table 3.1: The occurrence of *Caligus* spp. at different salinities of seven culturing *L. calcarifer* areas in Malaysia.

Type of culture	Location	Geographical location		System	Sampling date	Mean salinity (ppt)
		Latitude (N)	Longitude (L)			
Monoculture	Gelang Patah	1.436027	103.578415	Earthen pond	17-Nov-2009	14.30 ± 0.02
	Semantan	1.790995	109.790754	Floating cages	09-Oct-2009	24.52 ± 0.02
	Bkt Tambun	5.281964	100.396194	Floating cages	15-Oct-2009	28.30 ± 0.02
Polyculture	Semarahan	5.864468	102.496610	Floating cages	11-Nov-2009	5.48 ± 0.02
	Pendas	1.380785	103.642732	Floating cages	17-Nov-2009	28.49 ± 0.02
	Santubong	1.413165	110.202327	Floating cages	08-Oct-2009	25.43 ± 0.05

Table 3.2: Quantitative data for *C. chiastos*, *C. epidemicus* & *C. rotundigenitalis* collected during the study.

Location	No. of fish	Mean size of fish (range), cm	*C. chiastos*				*C. epidemicus*				*C. rotundigenitalis*			
			T	P	MA	MI	T	P	MA	MI	T	P	MA	MI
Gelang Patah	5	37 (35– 39)	-	-	-	-	138	100	27.6	27.6	-	-	-	-
Semantan	70	21 (19– 25)	-	-	-	-	17	-	0.24	-	-	-	-	-
Bkt Tambun	70	25 (21– 30)	1	1.4	0.01	1	11	11.43	0.15	1.38	4	2.86	0.05	2
Semarahan	70	20 (15– 25)	-	-	-	-	471	100	6.73	6.73	43	-	6.10	-
Pendas	70	24 (18– 26)	-	-	-	-	81	100	1.15	1.15	-	-	-	-
Santubong	41	26 (15– 30)	-	-	-	-	11	-	0.27	-	-	-	-	-

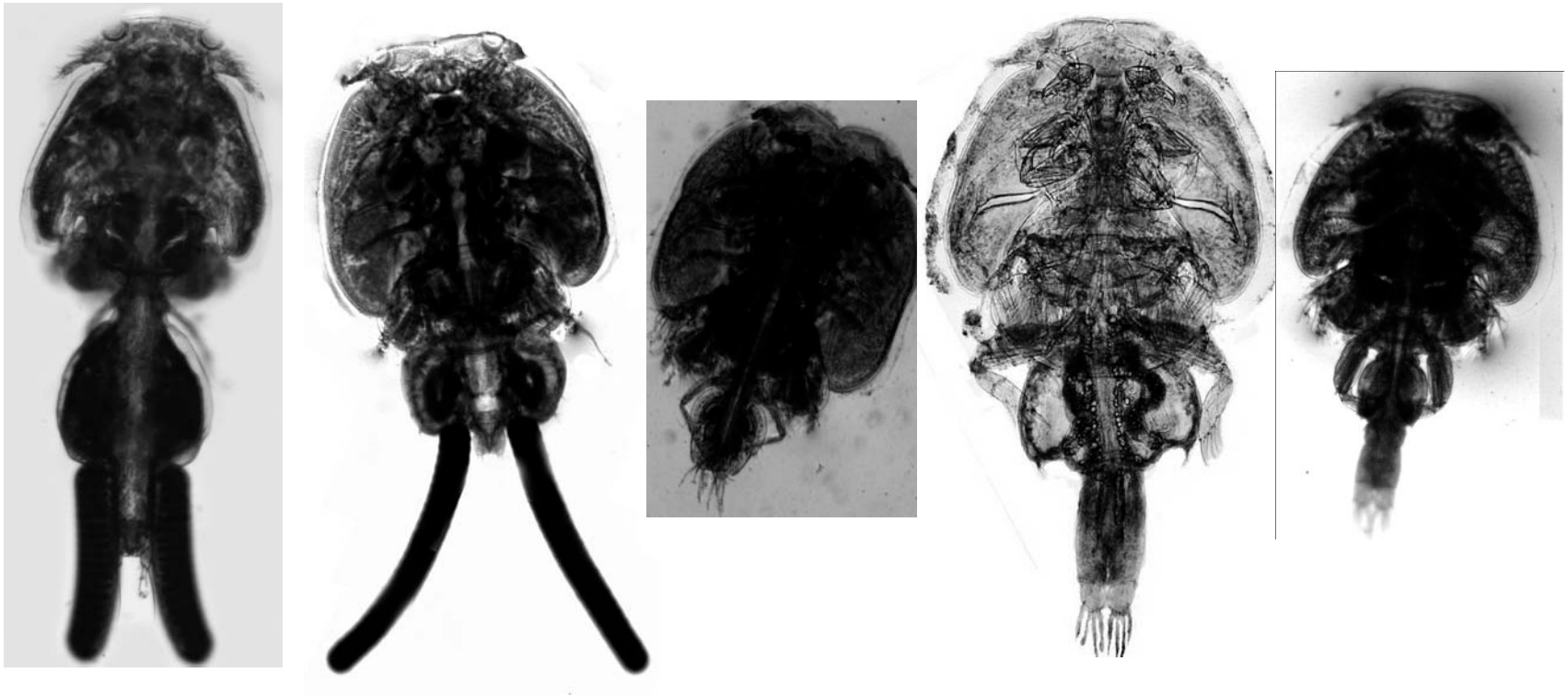

Figure 3.3: Microscopic observation of *Caligus* spp. isolated from cultured Asian seabass in Malaysia. A) Female *Caligus chiastos*, B) female *C. epidemicus*, C) male *C. epidemicus*, D) female *C. rotundigenitalis*, E) male *C. roundigenitalis.*

3.5 Discussions

The first disclosure of the parasitism of *Caligus* spp. on *L. calcarifer* in Malaysia was reported with the isolation of *C. epidemicus* in 2003, followed by *C. punctatus* three years later (Venmathi Maran *et al*., 2009). This present study revealed three additional species, *C. chiastos* and *C. rotundigenitalis* from the sampling in 2009. This addition accounts for a total of four species associated with the infection on the *L. calcarifer*. Among the species, *C. epidemicus* was the only species consistently isolated from the host throughout the timeline (2003 until 2009), in both culturing practices (mono and polyculture) and also at all observed water salinities.

This present study also revealed that *C. epidemicus* was the largest *Caligus* population infecting cultured *L. calcarifer* in Malaysia. This species accounts for 93.8% of the total isolations. The adaptability of this caligids to wider water salinity range was observed ranging from 5 to 28 ppt. This results support the report on the isolation of this species at salinity ranging from 4 to 28 ppt in Australia (Hewitt, 1971). On the other hand, it also indicated that the population growths activity of caligids which can cause more infection to the cultured fish. *Caligus epidemicus* is known to infect various cultured finfish in Philippines, cultured grouper in Vietnam, mullet and porgies in Taiwan and also snapper cultured in Malaysia (Leong and Wong, 1984; Ho *et al*., 2004; Vo *et al*., 2008; Kua and Faizul, 2010). Moreover, the infected hosts were not only restricted to fish species, the tiger prawn in Thailand was also vulnerable (Ruangpan and Kabata, 1984). Thus, due to such existence, this species is regarded as a killer species for fish farming activity in Asian continents (Venmathi Maran *et al*., 2009).

Despite accounting for less than 7% of the population size, the presence of the other two species may trigger for a potential threat to the industry. This is due to the fact that *C. chiastos* and *C. rotundigenitalis* are cosmopolitan species. Thus, they are known for the ability to adapt and proliferate onto different hosts. Currently, *C. chiastos* and *C. rotundigenitalis* have not been reported to infect *L. calcarifer* but closely has been associated with the infection on cultured snapper or grouper and several other species such as *Plectorhynchus cinctus* and *Thunnus maccoyii* for *C. chiastos* and *Mugil cephalus* and *Oreochromis mossambicus* for *C. rotundigenitalis* (Roubal *et al*., 1983; Lin and Ho, 1993; Lin and Ho, 2003; Yuniar *et al*., 2007; Hayward *et al*., 2008; Venmathi Maran *et al*., 2009).

This study also unveiled the vulnerability of *L. calcarifer* cultured in polyculture activity to the infections by the other species (Table 3.2). More species were discovered as compared to the opposite practice. This situation may have occurred due to a close interaction between the cultured stocks, while monoculture activity is able to minimize such contact. However, these results should be taken with caution since the presences of these caligids are directly affected by farm managements. Further studies are required in order to justify this assumption. In addition, the floating net cages also allowed the interaction between the cultured stocks with the wild-origin fish. This situation is one of the important areas that need to be highlighted, whether the parasitic organisms were either transmitted into the farm or out into the wild.

CHAPTER 4

Morphological Study of *Caligus* Muller, 1785 Parasitizing Cultured Asian Seabass, *Lates calcarifer*, Bloch 1790 in Malaysia

[All the data from this chapter has been submitted to Aquaculture Journal and presented at 8th Symposium on Diseases in Asian Aquaculture]

4.1 Introduction

Morphology-based analysis has lead to the fundamental discoveries of two *Caligus* Müller, 1785 species parasitizing cultured *L. calcarifer* in Malaysia (Appendix 1). They were identified as *C. epidemicus* Hewitt (1970) and *C. punctatus* Shiino (1955). These two species contributed to the overall parasitism of *Caligus* Müller, 1785 on cultured fin fishes along with *C. chiastos*, *C. longipedis* and *C. rotundigenitalis* (Appendix 1). The three latter species were closely related to *Lutjanus* spp. and *Ephinephelus* spp. Three out of the five species were regarded as killer species in the Asian continent; *C. epidemicus*, *C. rotundigenitalis* and *C. punctatus* (Venmathi Maran *et al*., 2009). Despite diversification in terms of host-parasite relationship, all of the species are considered as cosmopolitan for marine and brackish water farming industry (Lin *et al*., 1997; Ho *et al*., 2004; Vo *et al*., 2008; Venmathi-Maran *et al*., 2009).

This parasitic genus is classified under the sub-class Copepoda, order Siphonostomatoida and family Caligidae (Martin and Davis, 2001; Robert and Janovy, 2009). Basically, the taxonomic classification of *Caligus* spp is largely based on their morphological descriptions (Kabata, 1979; Huys and Boxshall, 1991). Candidates of this

genus shared quite similar morphology which are characterized by the four tagmata; cephalothorax, fourth leg-bearing segment, genital complex and the abdomen (Kearn, 2004). They have a flattened body and prehensile appendages, allowing them to attach to the host surface, as well as making them capable of free movement (Roberts and Janovy, 2009; Rohde, 2005). However, each species possess its own special characteristic in which distinguish them from the other taxa.

Chapter 3 has revealed parasitism of two additional species of *Caligus* spp. onto cultured *Lates calcarifer* in Malaysia; *C. chiastos* and *C. rotundigenitalis*. However, in the previous chapter, the identifications of these two species were primarily based on the observation under dissecting microscope (Leica Zoom 2000 and DM 5000) at eye piece magnifications of 50–400x. There were some critical points which were still unclear due to the low magnification power. Thus, in this chapter, the focal point is to have a clear and concise observation. Since, the primary stage of parasitology study is the discovery and description of the parasites itself (Robert and Janovy, 2009). This study would therefore provide the genuine structure of the isolated Malaysian species via Scanning Electron Microscopy (SEM). It involves evaluating the specific phenotypic criteria to re-examine the genus, family and order grouping of the parasite.

4.2 Methodology

4.2.1 Specimen collections

This chapter was an extension study derived from the previous chapter. Thus, the methodology for parasite collections was as discussed in Chapter 3 (Appendix 2; Table 3.1).

4.2.2 Sorting out the parasites

The isolated samples were sorted based on their basic morphological structures (Figure 3.2). Similar specimens were grouped together.

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4.2.3 Staining method (Alum-carmine)

A drop of alum-carmine was dropped onto the isolated *Caligus* spp. and left for 10 minutes and followed by dehydration process. The dehydration process involved a series of 50-90% ethanol to anhydrous alcohol with 15 minutes of holding time for every concentration. Then, one drop of olive oil was applied to the sample for cleaning purpose before it was mounted with DPX onto a glass slide.

4.2.4 Measurements and identifications

The stained specimens were observed and measured (length and width) under light microscopy (Leica Zoom 2000 and DM 5000) at magnification of 5-40X. Different species were separated and photographed. As for *C. chiastos*, the species was measured along with two other samples courtesy from the Malaysian Fisheries Research Institute (FRI), Penang. Just as discussed in Chapter 3, some samples were sent to Prof Ju Shey Ho from

Department of Biological Sciences, California State University, California for confirmation in identification.

4.2.5 Scanning electron miscroscopy (SEM)

Samples were fixed in *McDowell-Trump* fixative prepared in 0.1M phosphate buffer, pH 7.2 at 4°C for 24 hours. Next, it was postfixed in 1% Osmium tetroxide prepared in the same buffer as above at room temperature for 2 hours. After that, specimens were washed twice with distilled water, each washing step requiring 10 minutes of holding time. The sample was dehydrated with 50%, 75% and 95% ethanol with 15 minutes of holding time for every concentration. 100% ethanol is applied to the sample three times with 20 minutes of holding time. The dehydrated tissue was immersed in 2 ml of hexamethyldisilazane for 10 minutes. The hexamethyldisilazane is then decanted from the specimen vial and left in the dessicator to air dry at room temperature. Then, the dried specimen was mounted on to SEM specimen stub with a double-sided sticky tape. It was coated with gold before been viewed under LeoSupra 50VP Field emission SEM equipped with Oxford INCA400 energy dispersive x-ray microanalysis system at magnifications of 27X – 1.27KX.

4.3 Results

4.3.1 *Caligus chiastos* Lin and Ho, 2003

Only a single adult female specimen was isolated throughout the sampling (Figure 4.1).

4.3.1.1 Female adult

Overall, the total body length was 2.95 to 3.23 (3.09) mm. The cephalothorax shield was ovate, with length greater than width, 1.24 to 1.48 (1.36) x 1.11 to 1.31 (1.21) mm. The frontal plate was well developed with a pair of lunules and first antennae (Figure 4.1-A). As a typical siphonostomatoid, the mouth tube was carried in a folded position parallel to the body axis (Figure 4.1-B). The fourth leg bearing segment located at the posterior to the cephalothorax (Figure 4.1-C). Genital complex was sub-circular to oval, 0.62 to 0.84 (0.73) x 0.66 to 0.84 (0.75) mm (Figure 4.1-A). Abdomen has two segments, longer than wide, 0.22 to 0.50 (0.36) x 0.43 to 0.51 (0.47) mm (Figure 4.1-A). Each sac contained 24 (30-15) eggs.

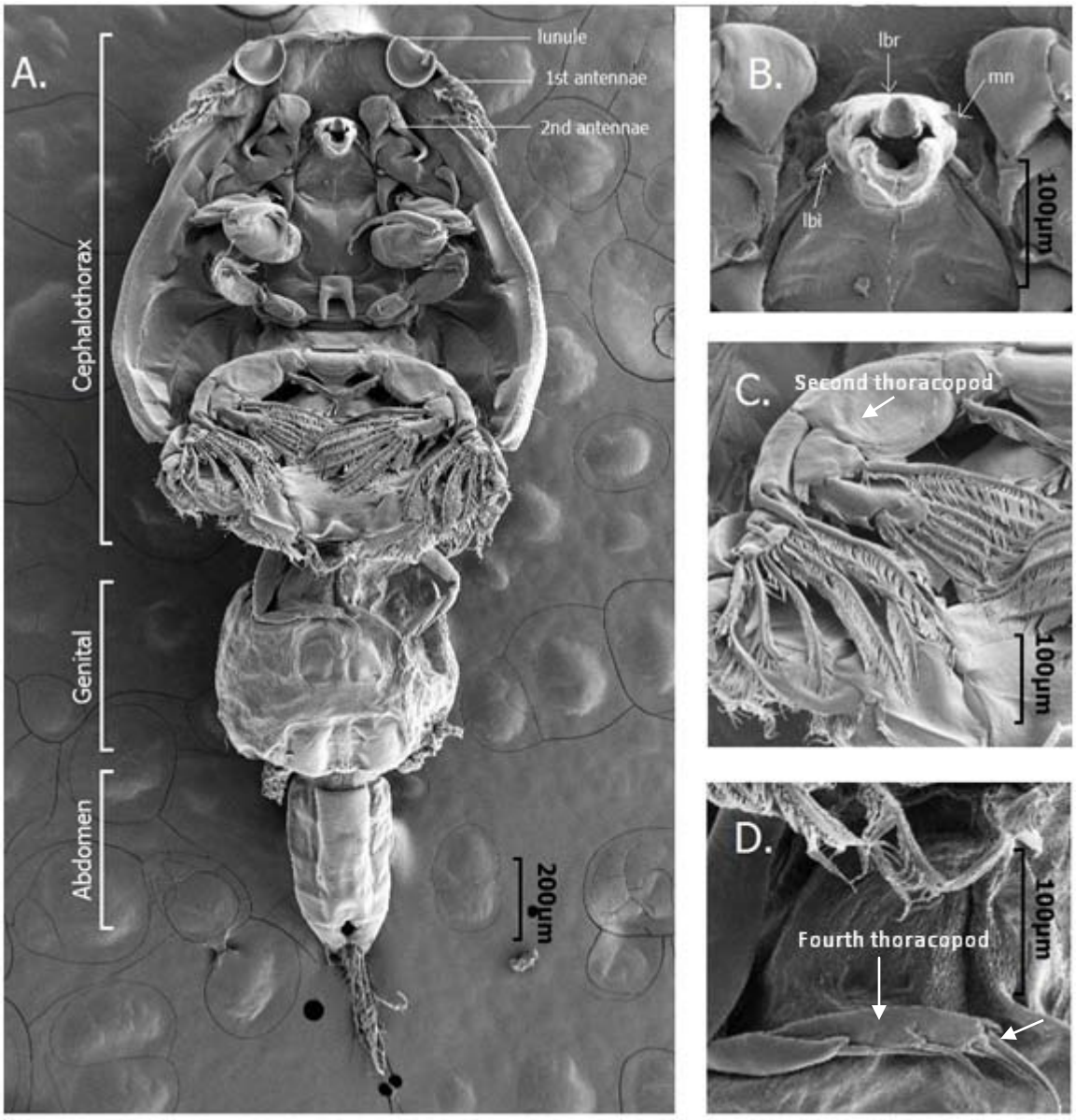

Figure 4.1: *Caligus chiastos* viewed under SEM. A: Ventral view of adult female without egg sacs; B: siphonostome mouth with labium, labrum and mandible *in situ*; C: second thoracopod and; D: four exopodal spines on fourth thoracopod. Magnifications: A=40X, B= 80X, C= 80X and D= 100X.

4.3.2 *Caligus epidemicus* Hewitt, 1970

SEM reveals that all of the samples shared the same bilaterally symmetrical structure (Figure 4.2). The total body length of female, 3.06 to 3.46 (3.26) mm, while male much shorter, 1.39 to 1.63 (1.51) mm.

4.3.2.1 Female adult

As for the female, the cephalothorax shield was sub-orbicular in shape, whereby both of their length and width were nearly equal, 1.76 to 2.08 (1.93) x 1.55 to 1.84 (1.66) mm (Figure 4.2-A). Similar to the adult male, the dorsal view shows that it was a smooth and roughly rounded surface (Figure 4.2-E). The genital segment was wider than the length, 0.53 to 0.74 (0.66) & 0.77 to 1.05 (0.99) mm respectively (Figure 4.2-A). All the edges were clearly broadly rounded. The abdomen was one-segmented and the size is 0.11 to 0.16 (0.14) x 0.15 to 0.19 (0.17) mm (Figure 4.2-A). It was very small and barely projecting beyond the genital segment. The female possesses a pair of egg sac which projects posterior to their genital segment. Each sac contained 24 (30-15) eggs (Figure 4.2-A).

4.2.2.2 Male adult

The overall structure of male was very similar to the female. The cephalothorax shield was sub-orbicular in shape, 1.31 to 1.17 (1.28) x 1.24 to 1.13 (1.19) mm (Figure 4.2-E). However, differences were observed in the structure and size of their genital segment and abdomen and also the absence of egg sacs. Under SEM, the genital segment possessed a cone-like shape (Figure 4.2-E). However, the live sample view suggested that this segment was sub-rectangular; 0.25 to 0.19 (0.22) x 0.30 to 0.23 (0.26) mm. The abdomen was sub-

quadrate and barely projecting beyond the genital segment (Figure 4.2-E). It was measured at 0.10 to 0.14 (0.12) x 0.15 to 0.15 (0.16) mm.

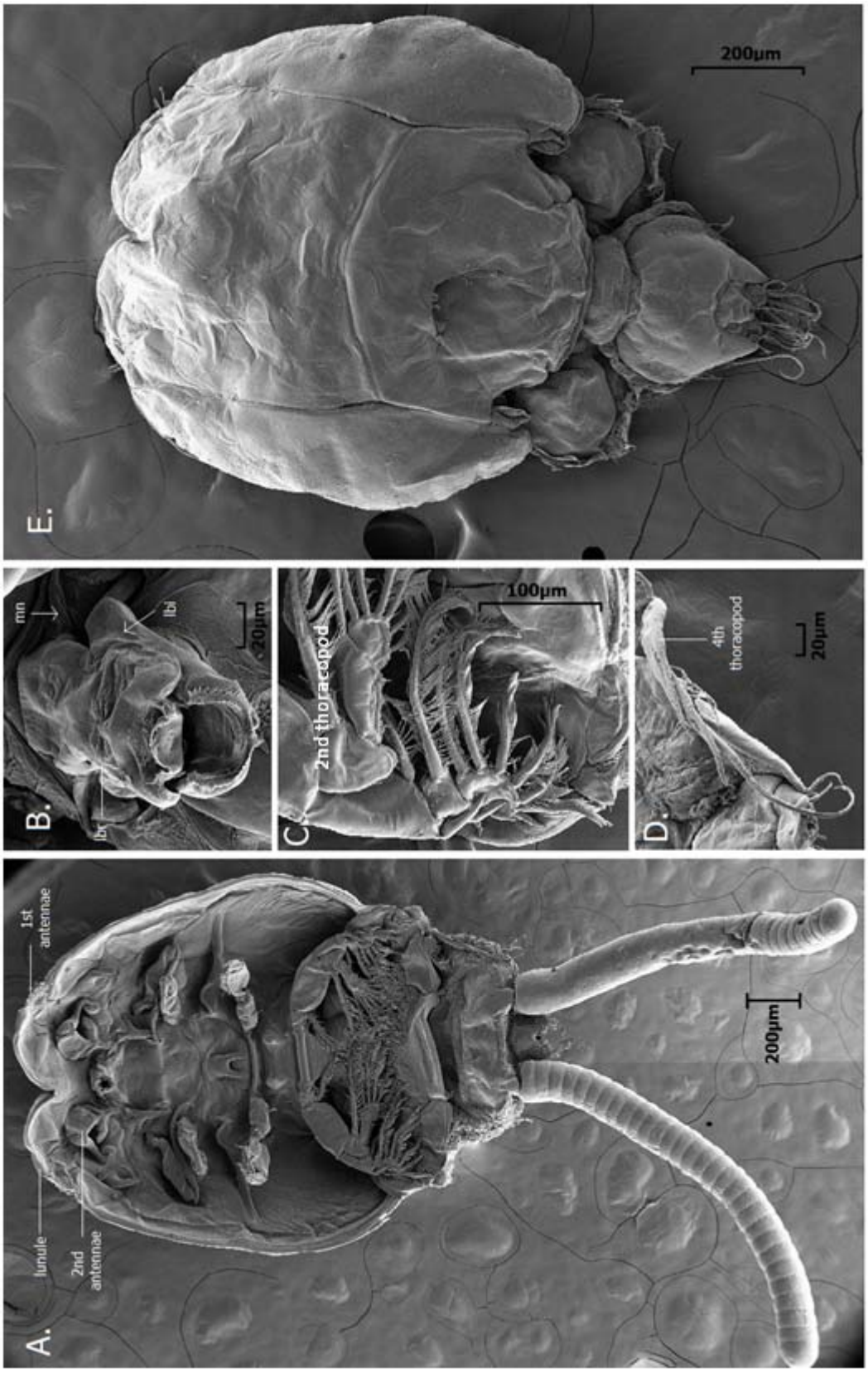

Figure 4.2: *C. epidemicus* Hewitt (1970) viewed under SEM. (A) Ventral structure of adult female with egg sacs, (B) siphonostome, mouth with labium (lbi), labrum (lbr) and mandible (mn) *in situ*, (C) second thoracopod, (D) exopodal spines of fourth thoracopod and (E) dorsal view of adult male. Magnification: A=34X, B=550X, C=154X, D=199X and E=69X.

4.3.3 *C. rotundigenitalis* Yü, 1933

The total length of female was absolutely longer than the male, 3.05 to 3.41 (3.23) mm as compared to 1.84 to 2.06 (1.90) mm. These measurements excluded the length of setae on caudal lamella.

4.3.3.1 Female adult

As for the female, the cephalothorax shield was sub-orbicular in shape, ranging from 1.21 to 1.61 (1.41) x 1.10 to 1.55 (1.33) mm (Figure 4.3-A). The structure of their genital segment is ovate in shape, measured from 0.56 to 0.82 (0.69) x 0.85 to 1.10 (0.97) mm (Figure 4.3-A). Under SEM, the abdomen was elongated and indistinctly two-segmented, 0.57 to 0.69 (0.63) x 0.25 to 0.34 (0.30) mm (Figure 4.3-A). Each egg sac contained with 26 to 30 (28) eggs.

4.3.3.2 Male adult

The male cephalothorax shield was also sub-orbicular, with wider than long, 1.08 x 0.92 mm (Figure 4.3-E). Their genital segment was smaller than the female. It was shorter than the thoracic zone of cephalothorax, with longer than broad, 0.33 x 0.26 mm. Similar to the female, the abdomen was two segmented, shorter than the genital complex and very indistinctly two segmented, 0.29 x 0.16 mm.

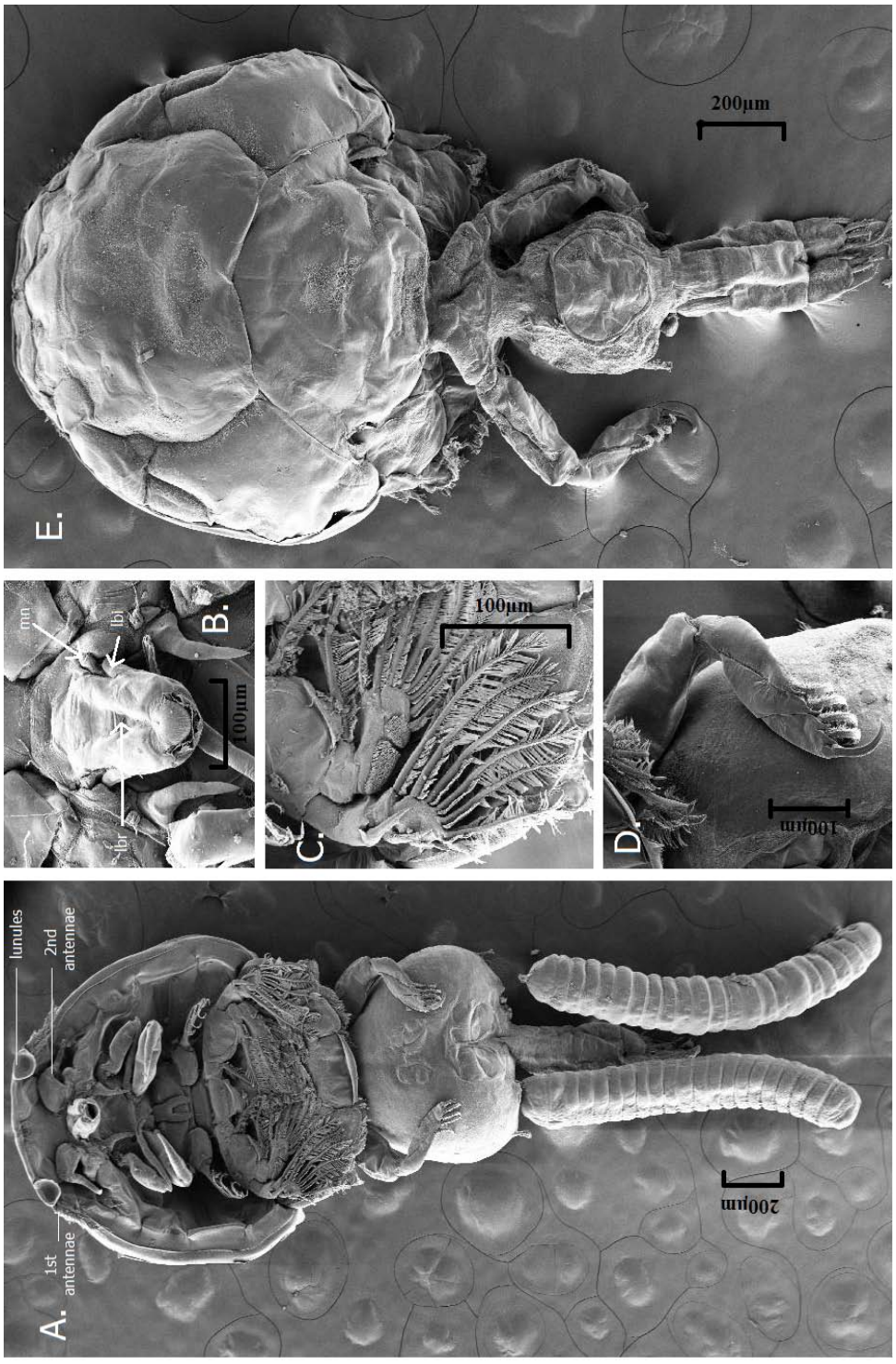

Figure 4.3: *C. rotundigenitalis* viewed under SEM. A: ventral view of adult female with egg sacs; B: siphonostome buccal cavity with labium (lbi), labrum (lbr) and mandible (mn) *in situ;* C: second thoracopod; D: endopod and exopod of fourth thoracopod and E: dorsal view of male. Magnification. A= 35X, B= 100X, C= 150X, D= 90X and E= 50X.

4.4 Discussions

SEM illustrates the distinctive morphological divergences in between the isolated Malaysian species. Their structures showed no divergences from the previous published literature. The three species were largely defined by the structure of their tagmata; cephalothorax, fourth thoracopod, genital segment and abdomen. Each of the characterized tagmata holds in a unique structure, which is a clear identifications tool. In comparison, the cephalothorax structure of *C. chiastos* is ovate while the other two species are sub-orbicular in shape. The same structure was observed in between the male and female of the same species.

Through SEM, the structures of their fourth thoracopod were able to differentiate the three species clearly. *C. rotundigenitalis* holds in with the largest fourth thoracopod among the three species (Fig. 4.3-D). It consists of one-segmented endopod and three-segmented exopod. The exopod is equipped with five equally length spines; proximal and middle segment with one spine each, while the distal segment with three spines. *Caligus chiastos* have a pair of thinner fourth leg. It consists with one-segmented endopod and two-segmented exopod. Their endopod is armed with four-spine and the apical spine is the longest. The third species (*C. epidemicus*) have a set of thin and most elongated fourth leg. It is armed with only two spines and the apical is the longest. As similar with cephalothorax, the structure is similar between sexes.

The species is differing on the structures of their abdomen. *C. epidemicus* tend to have the shortest abdomen; it is sub-quadrate and barely projects beyond genital segment. *C. chiasos* equipped with an elongated abdomen similar to *C. rotundigenitalis*. Structural

differences in between sexes can be observed in the structures of the genital segment. Male tend to have a smaller and shorter tagma than the female. This sexual divergence is constantly observed in all species. The male holds with much smaller genital segment as compared to the female (Fig. 4.1, 4.2 and 4.3). However, this structural divergence is just on the size, the basic for both sexes are still the same; *C. chiasos* holds in with sub-orbicular, *C. epidemicus* with sub-rectangular and *C. rotundigenitalis* with ovate structures of genital segment (male and female).

According to Venmathi Maran *et al*. (2009), the most distinctive attribute which differentiates *C. chiastos* among the other members of its genera is the structure of their exopodal spine on second thoracopod (Fig. 4.1-C) and the presence of four exopodal spines on their two-segmented fourth thoracopod (Fig. 4.1-D). As for *C. epidemicus*, it relies on the structure of their thin and well elongated fourth thoracopod (Fig. 4.2-D). The non-segmented endopod was longer than the two-segmented exopod. The first exopodal segment consists of one single spine and the terminal segment with two spines; one long setae and shorter distal spine.

Besides all of the variations, the similarities among the specimens clarified them under the same well-accepted taxonomic classification. This is the important point in which classified these species into the same ordinal. Based on the SEM results, each of the isolated species holds in the same basic structure as their primitive hypothesized ancestor; *Cyclops* sp (Rohde, 2005). Typically, they are classified as Crustacea by the presence of two sets of antennae, cephalothorax, free thorax and the abdomen. On the basis of Copepoda, Huys and Boxshall (1991) hypothesized that the copepods are generally small in

size; 0.5 to 5 mm. The first, second and third thoracopods were fused altogether with cephalothorax shield.

Caligus is known to possess a podoplean-type body structure (Rohde, 2005). It is a distinguishing characteristic in which differentiates podoplean from the members of gymnoplean; Calanoida and Platycopoida (Kabata, 1979). However, oligomerization is observed on *Caligus* as it only possessed with one instead of five segments in between the cephalothorax shield and genital segment. Besides, the first two thoracopod was attached dorsal of the cephalothorax shield. *Caligus* is defined as siphonostomatoid by the presence of distinctive tubular mouth apparatus or siphonostome mouth. This type of mouth part is characterized by the elongated and tapering structure. Besides that, it is equipped with the elongated mandibles which are enclosed within the siphon (Kabata, 1992). However, the mandible structure is not visualized under SEM since it is incorporated inside a tubular mouth cone.

Table 4.1: Summary of morphological structures of the isolates

Criteria	*C. chiastos*	*C. epidemicus*		*C. rotundigenitalis*	
	Female	**Female**	**Male**	**Female**	**Male**
Total length (mm)	2.95- 3.23 (3.09)	3.06- 3.46 (3.26)	1.39- 1.63 (1.51)	3.05- 3.41 (3.23)	1.84- 2.06 (1.90)
Cephalothorax	Ovate	Sub-orbicular	Sub-orbicular	Sub-orbicular	Sub-orbicular
4th thoracopod	-Thin -Exopod 3-segmented -Armed with 5 spines -Apical spines longest	-Thin -Exopod 2-segmented -Armed with 3 spines -Apical spine longest	Similar with female	-Large -Exopod 3-segmented -Armed with 5 spines -Spines length equally	Similar with female
Genital segment	Sub-orbicular	Sub-rectangular	-Sub-rectangular -Smaller than female	Ovate	-Ovate -Smaller than female
Abdomen	-2-segmented -Longer than wide - Projecting beyond GS	-Small sub-quadrate -Barely projecting beyond GS	Similar with female	-1-segmented -Longer than wide -Projecting beyond	Similar with female

CHAPTER 5

Phylogenetic Study of *Caligus* Muller, 1785 Parasitizing Cultured Asian Seabass, *Lates calcarifer*, Bloch 1790 in Malaysia

[All the data from this chapter has been submitted to Aquaculture Journal and presented at 8th Symposium on Diseases in Asian Aquaculture]

5.1 Introduction

The host range of *Caligus* Müller (1785) is one of the areas of interest due to large diversification. Some species are highly regarded as host-specific and some species which are not. To date, the Malaysian-isolate caligids were all cosmopolitan (Lin *et al*., 1997; Ho *et al*., 2004; Vo *et al*., 2008; Venmathi Maran *et al*., 2009). Due to this factor, identification based on host-parasite relationship is difficult to employ. On *L. calcarifer*, the infection was pointed by two species; *C. epidemicus* Hewitt (1970) and *C. punctatus* Shiino (1955) (Venmathi-Maran *et al*., 2009). These two species contributed to the overall parasitism on cultured fin fishes along with *C. chiastos*, *C. longipedis* and *C. rotundigenitalis*.

The evaluation regarding on the parasitism of *Caligus* in Malaysia was defined solely through morphology-based perspective. This identification viewpoint was discussed in previous chapters. There is still no clarification in terms of other biological view points. Such clarifications are profoundly needed since the knowledge would likely to generate for a greater understanding.

In terms of molecular phylogenetic studies, they are yet to be investigated. Most studies are mainly focusing on *C. curtus*, *C. elongatus* and also *Lepeophtheirus* spp. For

this purpose, the ribosomal RNA region is considered to be an appropriate reference in resolving phylogenetic relationship. This is due to their conserved and slow evolving characteristic (Martin *et al*., 2009). The reliability of 18S region for crustacean phylogeny has been questioned due to the insufficient variability in the sequences (Martin and Davis, 2009). On the other hand, 28S rRNA gene consists of a larger sequence site, thus the expansion regions are much more variable (Hancock *et al*., 1988).

28S rRNA analysis of *C. elongatus* Nordmann, 1832 collected from south-eastern coast of Norway have suggested that the species itself have two distinct genotypes (Øines and Schram, 2008). Besides that, a recent study on the D1-D2 region of the gene suggesting that Australian isolate *C. chiastos* is grouped with *C. elongatus* in the same monophyletic clade (Nowak *et al*., 2011). Thus, this chapter emphasized the third objective of this present study. The focus is to disentangle the phylogenetic information inferred based on the 28rRNA gene of *C. chiastos*, *C. epidemicus* and *C. rotundigenitalis.* It was compared with other available Copepoda taxa retrieved from GenBank™ .

5.2 Methodology

5.2.1 Specimens collections and sorting out

The methodology for parasite collections was as discussed in Chapter 3 (Appendix1; Table 1). The isolated samples were sorted based on their basic morphological structures (Figure 3.3). Similar specimens were grouped together and preserved in 70% ethanol and stored in -20˚C until used.

5.2.2 Genomic DNA extraction

Specimens fixed in 70% alcohol were subjected to molecular analysis. At first, the samples were extracted using the DNeasy® Blood and Tissue Kit (QIAGEN Inc.) according to the manufacturer's protocol. Then, the concentration of total genomic DNA was determined using NanoDrop spectrophotometer.

5.2.3 Polymerase Chain Reaction (PCR)

1 ng of DNA sample was amplified in 20 µl Maxime PCR Premix kit, i-Taq (iNtRON Biotech) reaction mixture with 5 pmol of primers (Table 5.1). The primers for 28S rRNA gene amplifications were designed from sequence retrieved from GenBank™ (DQ180337.1). PCR reactions were performed in a MyCycler™ Thermal Cycler (Bio-Rad, US). The thermal cycling program was as follows: 2 min initial denaturation at 94°C, followed by 30 cycles of 20 s of denaturation at 94°C, 10 s of primer annealing at 55°C, and 40 s extension at 72°C, and 5 min at 72°C for a final extension. As for primer 3, the annealing temperature was set to 55.7 °C.

Table 5.1: Primers used to amplify Malaysian isolates *Caligus* spp 28S rRNA gene. Primers generated from Primer 3 software.

No.	Primer Name	Primer Sequence (5' to 3')
1	28S-1F	TGA ACA GGG TAA AGC CCA TCA C
	28S-1R	GGA TGG TGT AAA CGA AAG ATG
2	28S-2F	GGG TAA AGC CCA TCA CTG AA
	28S-2R	AGA GCA TAC ACG TTG GGA CC
3	28S-3F	CGG CTT GAG ATC TTT TGA GC
	28S-3R	ACG AGT AGG AAG GTC GCA GA
4	28S-4F	GTA GCG ATT CTG ACG TGC AA
	28S-4R	AAT CAA AGC CGA AAG GGA AT
5	28S-5F	ACG AGT AGG AAG GTC GCA GA
	28S-5R	TGG ATC ACC TTA GCT TCG CT
6	28S-6F	GGG AGA ATG GGT TTT GGT TT
	28S-6R	ATT GTG GAG GAG CAT CTT
7	28S-7F	CGA GAT TCC CAC TGT CCC TA
	28S-7R	TTG TTC ACC CGC TAA TAG GG
8	28S-8F	GGG CGT AGA ATA GGG AGG AG
	28S-8R	TTG TTC ACC CGC TAA TAG GG

5.2.4 Gel Extraction

The final PCR products were separated on 1.2% Agarose gel for 45 min. The targeted bands were excised and purified using QIAquick® Agarose Gel Extraction Kit (QIAGEN Inc.). The concentration of the extracted samples were determined and sent for direct sequencing.

5.2.5 DNA sequence assembly and alignment

Final sequence (query) was subjected to sequence similarity searches using BLASTn algorithm against the non-redundant database retrieved from NCBI (http://www.ncbi.nlm.nih.gov). Several reference taxa were selected (Table 5.2) and aligned altogether with the query sequence using Mega 4.0 software (Swofford, 2002). The alignment was trimmed according to the size of query sequence.

5.2.6 Phylogenetic analysis

Three phylogenetic inference methods were used; maximum likelihood (ML), maximum parsimony (MP) and neighbor-joining (NJ). All methods were computed using PAUP* 4.0 software (Swafford, 2002) via heuristic search using bisection-reconnection (TBR) branch swapping algorithm and random sequence addition. MP analysis utilized the parsimony informative sites and NJ analysis was carried using Kimura 2-parameter. In ML analysis, the GTR+G substitution model was chosen as the best evolutionary model according to Akaike Information Criterion (AIC) in Modeltest 3.7 (Posada and Crandall, 1998). This analysis was computed with 100 bootstrap replicates. Statistical support for the internal branches in MP and NJ was evaluated by bootstrap analysis based on 1000 replications.

5.3 Results

5.3.1 Information of amplified gene

The amplification of 28S rRNA gene yielded fragments consisting with 3096 bp for *C. chiastos* and *C. epidemicus* and 3120 bp *C. rotundigenitalis*. The G+C content of each species was 47.7 %, 47.9% and 47.9%. 21 taxa which originated from five different orders (Calanoida, Cyclopoida, Harpacticoida, Siphonostomatoida and Poecilostomatoida) were selected from BLASTn searches result (Table 5.2). *Marchilis hrabei* (EF199981.2) was selected as the out-group. BLASTn results simplify that *C. chiastos* and *C. rotundigenitalis* have greater homology with *C. elongatus* while *C. epidemicus* closer with *C. curtus*. *Lepeophtheirus* spp candidates come in with second-best scoring result (Table 5.2).

Table 5.2: List of 21 selected taxa originated from five Copepoda order based on BLASTn searches result.

Order	Family	Taxon	Accession number	Size (bp)
Siphonostomatoida	Caligidae	*Caligus curtus*	DQ180338.1	3150
		Caligus elongatus	DQ180337.1	3166
		Caligus elongatus	DQ180336.1	3141
		Lepeophtheirus pollachius	DQ180343.1	3156
		Lepeophtheirus salmonis	DQ297550.1	3193
		Lepeophtheirus salmonis	DQ180342.1	3692
		Lepeophtheirus salmonis	EU929084.1	7061
	Lernaepodidae	*Neobrachiella merluccii*	DQ180347.1	3133
		Clavella stellata	DQ180339.1	3138
		Salmincola edwardsi	DQ180346.2	3136
	Sphyriidae	*Sphyrion lumpii*	DQ180345.2	3305
	Pandaridae	*Echtrogaleus coleoptratus*	DQ180344.1	3202
Harpacticoida	Canuellidae	*Canuella perplexa*	EU370445.1	3462
	Harpacticidae	*Tigriopus cf. fulvus*	EU370444.1	3532
Cyclopoida	Cyclopidae	*Cyclops insignis*	EF532821.2	5729
		Cyclops kolensis	EF532820.2	6008
		Cyclopidae sp.	AY210813.1	3536
	Cyclopettidae	*Paracyclopina nana*	FJ214952.1	7974
Calanoida	Calanidae	*Calanus simillimus*	EU914255.1	3243
Poecilostomatoida	Chondracanthidae	*Chondracanthus lophii*	DQ180341.2	3411
		Chondracanthus merluccii	DQ180340.1	3320

4.3.3 Alignment (ClustalW)

Sequence alignment disclosed with a total of 3513 nucleotide sites (including gaps). Out of all, only 1053 sites were known to be parsimony informative and 1863 sites were conserved. Overall genetic distances between all of the selected taxa are summarized in Appendix C. It is found that the mean average sequence divergence within the selected members of *Caligus* is 0.04%. *Caligus chiastos* was closer to *C. elongatus* instead of the other two isolated species in this present study. *C. epidemicus* and *C. rotundigenitalis* were closer to *C. curtus*. The average divergences between the selected Caligidae are denoted in Table 5.3.

5.3.2 Phylogenetic analysis

Phylogenetic analyses of 28S rRNA have constantly recapitulated the monophyletic relationship of *C. chiastos*, *C. epidemicus* and *C. rotundigenitalis* with all Caligidae (Figure 5.1). It appears that *C. rotundigenitalis* branched out from the *C. chiastos*-*C. elongatus* monophyletic group and *C. epidemicus* was the most primitive candidate of *Caligus*. It was noted that the topology of both NJ and ML were compatible with the ordinary classifications since all of the isolated species were incorporated into the genus *Caligus* as expected (Figure 5.1-A, B). In contrast, MP analysis placed *C. epidemicus* independently outside either *Caligus* or *Lepeophtheirus* group (Figure 5.1-C).

The branching pattern of *C. rotundigenitalis* was also inconsistently been inferred. The species appears to evolve from *C. curtus* in MP, whereby NJ and ML inferred the other way. These variations might be derived from the differences in methods used in constructing the trees. All of the selected orders (I-V) are apparently distinctive groups of Copepoda which were well-separated from Hexapoda; *M. hrabei*.

In between this Copepoda lineage, Calanoida (V) appears to be the most ancient group. It forms in as the basal taxon to the branch arrangement for the whole Copepoda. It was followed by Cyclopoida (IV) branched out from the major group of Siphonostomatoida-Poecilostomatoida-Harpacticoida (I-II-III). It was illustrated that Poecilostomatoida (II) was the closest relative to Siphonostomatoida (I), followed by Harpacticoida (III). This arrangement was constantly reproduced by all of the analyses (Figure 5.1).

Table 5.3: The estimated average of evolutionary divergence between the selected taxa of Caligidae based on the partially amplified 28S rRNA sequences.

Taxon	1	2	3	4	5	6	7	8
C. chiastos								
C. elongatus (DQ180336.1)	0.016							
C. elongatus (DQ180337.1)	0.016	0						
C. rotundigenitalis	0.035	0.032	0.032					
C. curtus (DQ180338.1)	0.035	0.028	0.028	0.036				
C. epidemicus	0.044	0.043	0.043	0.046	0.042			
L. pollachius (DQ180343.1)	0.047	0.045	0.045	0.040	0.041	0.044		
L. salmonis (DQ180342.1)	0.052	0.046	0.046	0.047	0.041	0.050	0.021	
L. salmonis (EU929084.1)	0.052	0.046	0.046	0.047	0.041	0.050	0.021	0

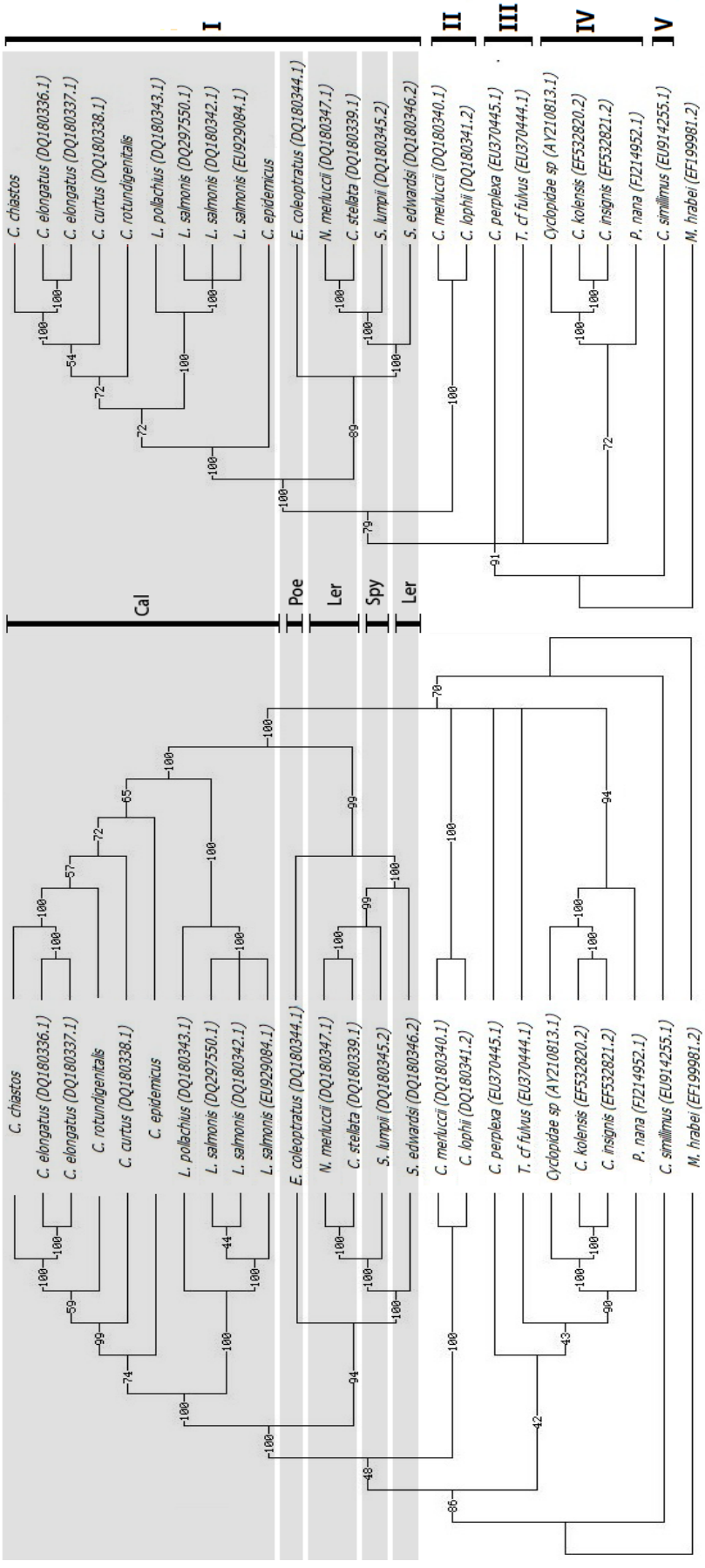

Figure 5.1: Bootstrap consensus tree derived from partial 28S rDNA sequences of *C. chiastos*, *C. epidemicus* and *C. rotundigenitalis* against 21 Copepoda taxa retrieved from the GenBank™. These taxa were originated from five orders (I-V); I= Siphonostomatoida, II= Poecilostomatoida, III= Harpacticoida, IV= Cyclopoida and V= Calanoida. Under Siphonostoimatoida there were families; Cal=Caligidae, Poe= Pandaridae, Ler= Lernaepodidae and Spy= Sphyriidae. These taxa were inferred using three phylogenetic methods; A: neighbour-joining (NJ); B: maximum likelihood (ML) and C: maximum parsimony (MP). Bootstrap values are noted on the branch.

5.4 Discussion

Lates calcarifer culturing industry in Malaysia is susceptible to the invasion of four species of *Caligus* Müller (1785); three species from this present study and *C. punctatus* in Venmathi Maran *et al*. (2009). In terms of behaviour, these parasitic organisms share the same cosmopolitan characteristic. They are known to parasitize several different species of cultured fin fish. Their geographical distribution is widespread within the Asian continent as well as other non-tropical climate area such as southern Australia. Besides that, the relationship in between the species lies on the similarity of their morphology. This is the important point which classified these species into the same ordinal.

The underlying interrelationship of Malaysian isolates through the genecology perspectives is illustrated in this present study. Besides, the sequences analysis and construction of simulated phylogenetic trees presented in this study unveils the connection between the isolates with other Copeopoda taxa throughout the globe (Fig. 4). Although the separation was determined by utilizing limited taxa, this study would provide as a stepping stone for further explorations. The analyses which are derived from partial 28S rRNA sequences reflect the morphology-based classification of the isolated species; family Caligidae, order Siphonostomatoid and subclass Copepoda. The gene was able to differentiate between *Caligus* Müller (1785) from *Lepeophtheirus* von Nordmann (1832) and simplify the Caligidae families. However, it is important to be noted that despite being highly supported by statistical analysis, the generated results should be cautiously taken.

The traditional morphology perspective has hypothesized that both *Lepeophtheirus* von Nordmann (1832) and *Caligus* Müller (1785) are two well-separated genera within the Caligidae family. Morphologically, they are differing in terms of the presence of a pair of lunules at the anterior portion of their frontal plate (Hopla et al., 1994; Boxaspen, 2006; Roberts and Janovy, 2009). This type of classification is in agreement with the study that *Caligus* formed a monophyletic group with another two *Caligus* taxa; *C. elongatus* and *C. curtus*. The branching patterns in both NJ and MP analyses constantly support this result. Furthermore, this *Caligus* Müller (1785) cluster forms a sister-group relationship with *Lepeophtheirus* von Nordmann (1832) (*L. salmonis* and *L. pollachius*) within the Caligidae monophyletic lineage (Fig. 5).

Caligus chiastos appears to have a closer genetic relationship with *C. elongatus* (Fig. 5). Nowak *et al*. (2011) also suggesting that the two species were incorporated within the same monophyletic linkage. The inferred phylogenetic tree shows that *C. rotundigenitalis* is closer to *C. curtus*. It shows that *C. epidemicus* tend to be more ancient origin, followed by an evolution into *C. curtus* and finally *C. elongatus*.

The Caligidae (*Caligus-Lepeophtheirus*) cluster is well-separated from the other three Siphonostomatoida families retrieved from the GenBank (Fig. 5). However, from the trees, the conspicuous area is within the relationship of Lernaeopodidae and Sphyriidae. It seems like both *C. stellata* and *M. merlucii* (Lernaeopodidae) evolved from *S. lumpii* (Spyriidae). 100% of bootstrap score were observed in both phylogenetic trees. This type of relationship supports the suggestion by Kabata (1992), where these two families were closely related. The assumption is supported by the similarity in the

morphological structures of their male candidates. Based on the morphological descriptions, the male is oval and un-segmented (Kabata, 1992).

In larger data set of Copepoda, the current analyses resulted in a similar topology as proposed by Huys and Boxhall (1991) and Razoul and Raibout (1996). These two published literatures complemented each other. They summarized the formation of monophyletic clade of Mormonilloida, Harpacticoida, Poecilostomatoida, Siphonostomatoida and Monstrilloida. The Cyclopoida forms sister-group to the clade, while Calanoida and Platycoida formed in a separated group basal to the others. The present analyse however comprises of only five of the retrieved orders; Harpacticoida, Poecilostomatoida, Siphonostomatoida, Cyclopoida and Calanoida. Each order forming their own monophyletic group, in which structured similarly as previous published literatures.

In terms of the 28S rRNA analysis, evaluation of trees in this study is dissimilar with Braga *et al*. (1999) since the calanoids forms as a basal to Copepoda. However, it is important to note that Braga *et al*. (1999) utilized the D9/D10 segments of the 28S rRNA instead of focusing the whole gene. It is known that high degrees of diversity were observed in phylogenetic analyses of arthropoda and crustaceans (Turbeville *et al*., 1991; Giribet and Ribera, 2000; Mallatt *et al*., 2004). The uncertainty of Copepoda phylogenies is still debatable due to the discrepancies between several published literatures, regardless the 18S, 28S or the mitochondrial genes. The sequences arrangement, signal and phylogenetic analysis of the 28S rRNA sequences are differing in between species.

Principally, precise phylogenetic analyses are corresponding to the stability and accuracy of taxonomic classifications. Besides, it is often been concluded within arthropod phylogeny based on rRNA sequences, more taxa or more data might result in higher tree resolution (Spears and Abele, 1997). In this study, it was observed that the exploration of *Caligus* Müller (1785) and as well as the Copepoda are still very far from complete. As known, *Caligus* is a genus which regarded as the largest of Copepoda whereby, it is comprises with more than 250 species. However, only *C. elongatus* and *C. curtus* are appropriate to include in this study. Noticeably, there are several other taxa such *as C. warlandi,* available in the GenBank™ have to be left out since the study was exploring the whole partial sequences of Malaysian isolate *Caligus*.

CHAPTER 6

General Discussion

Previous chapters have thoroughly discussed on a series of vital circumstances, literatures and results which related to the parasitism of *Caligus* spp. onto cultured Asian seabass in Malaysia. However, as discussed, the documentation is limited in Malaysia. Strategized by three major objectives, this present study would likely to fill in some of the remaining puzzles and further fish out for new ideas. The major upshot of this study is more on unveiling the causative parasite. This would directly figures for greater understanding on their negative impact onto the aquaculture industry, it includes; the fish health issues, subsequent economic defect, transmission of the parasite, host-switching capability, adaptability and parasite population structure. Besides, further advance phylogenetic analysis was also included.

6.1 Abundance of infections

The documentation regarding the abundance of infections has initiated breakthrough information regarding the major infection by *C. epidemicus*. Results have shown that this species relatively accounts for up to 98.6% of the total isolations. It was present at all sampling locations with the highest abundance, regardless of the differences in the geographical areas, water salinities, types of culturing and systems. These results proved that the cosmopolitan characteristic of by the species is not only restricted to the host-parasite relationship. This species seems to be cosmopolitan towards all of the observed disparities; either natural or man-made circumstances (Table 3.2). In terms of survivability, the collected data illustrate that this species is capable to withstand the fluctuation of water

salinities at a very wide range (4 to 28 ppt). The abundance of *C. epidemicus* in the Malaysian aquaculture industry should be taken seriously since this species is listed among the killer species in Asia. It was related to the mass mortality of cultured fish in Taiwan (Lin and Ho, 1994). This species is known to be infectious for various types of fin fishes, right from South East Asia down to the southern part of Australia. Besides fin fishes, it is also known to parasitize cultured black tiger shrimp, *Penaeus monodon* in Thailand (Ruangpan and Kabata, 1984). The presence of *C. epidemicus* was first reported in Malaysia by Venmathi Maran *et al*. (2009).

This objective also resulted in the first discovery of two additional species of *Caligus* parasitizing the host in Malaysia; *C. chiastos* and *C. rotundigenitalis*. As discussed in Chapter 3, the number of *Caligus* species infecting *L. calcarifer* increased up to four with this addition. Despite their presence in low numbers, the impact of these species should be highlighted since they are able to infect *L. calcarifer* cultured particularly at salinity between 25 to 28 ppt (Table 3.2). Besides, the data would likely show how vulnerable the polyculture industry is towards the infection by *Caligus*. Despite all of the management followed by the farm, the presence of *Caligus* is becoming more evident. Moreover, one of the species is regarded as a killer species in Asia; *C. rotundigenitalis*. *Caligus chiastos*) has been labeled as a rare species since it was found in small numbers in various occasions, including the first report in Malaysia (Roubal *et al*., 1983; Lin and Ho, 2003; Venmathi Maran *et al*., 2000). This present study also pictured the same situation whereby the one specimen of this species was isolated (Table 3.2). However, the situation at southern area of Australia is someway different, *C. chiastos* is acknowledged to be the predominant causative species (Hayward *et al*., 2010).

As discussed in Chapter 3, *C. epidemicus* was able to withstand over wide range of water salinities (Table 3.2). The same scenario was observed in Australia (Hewitt, 1971). As for *C. chiastos*, this species was only present at approximately 28 ppt in polyculture farm. In Australia, it was found parasitizing marine farmed southern blue fin tuna, *Thunnus maccoyyi*. Finally, *C. rotundigenitalis* is more known for their infection at brackish water farming industry. In this study, the presence of this species was recorded at approximately 28 ppt (Table 3.2). It was found to infect several fin fish in Taiwan, Indonesia and Malaysia.

All reported cases related with the parasitism of *C. chiastos, C. epidemicus* and *C. rotundigenitalis* cover within the tropical ocean of Asian countries and also the temperate ocean of southern part of Australia. This situation triggered for an interesting question; how they survive the climate changes? As known, Southern Australia is a temperate region with four different seasons, thus the fluctuation of sea temperature is varied greatly. Meanwhile, Asian continents appear to have more constant temperatures to compare with the temperate because only affected by the dry and wet seasons. It has been speculated that decreasing in temperature would able to reduce growth and productivity of *C. chiastos* (Hayward *et al*., 2008). However, a study conducted on Degen's leatherjacket, *Thamnaconus degeni* shows that the speculation is not significant (Hayward *et al*., 2011). Thus, this area of research is one of an interesting area to be explored in the future since it is not included in the present study.

The major concern rise from this objective is the capability of these present species to infect other aquatic organisms within the culturing areas; either cultured or wild populations. This is due to fact that all of the isolated species are well known for their low-host specificity. This often means that they can infect other types of hosts which are present in the same area. There are vast literatures that highlight the probability for this circumstance to happen. As known, the parasite is able to well-propagate in aquaculture system due to the massive protein source (host) provided by the industry. Thus, the parasite population would likely to expand if there is no proper strategy to stop this proliferation.

6.2 Morphological studies

SEM has successfully revealed the genuine structures of *C. chiastos*, *C. epidemicus* and *C. rotundigenitalis*. It supports the descriptions by Venmathi Maran *et al*. (2009). The results enable this study to confirm that *L. calcarifer* was also susceptible to the infection by *C. chiastos* and *C. rotundigenitalis*. *Caligus epidemicus* has been commonly found infecting the studied host, but different situation was observed for *C. chiastos* and *C. rotundigenitalis*. These two later species are known for their parasitism onto cultured snapper in Malaysia and this is the first report on cultured Asian seabass.

According to Venmathi Maran *et al*. (2009), the most distinctive attribute which differentiates *C. chiastos* among the other members of its genera is the structure of its exopodal spine on the second thoracopod (Fig. 4.1-C) and the presence of four exopodal spines on their two-segmented fourth thoracopod (Fig. 4.1-D). As for *C. epidemicus*, it relies on the structure of their thin and well elongated fourth thoracopod (Fig. 4.2-D). The non-segmented endopod was longer than the two-segmented exopod. The first exopodal

segment consists of one single spine and the terminal segment with two spines; one long setae and shorter distal spine.

C. rotundigenitalis was first reported in Malaysia by Leong (1984). It was reported to infect silver pomfret, *Pampus chinensis*. However, Ho *et al*. (2000) have confirmed that it has been misidentified and the actual causative species was *C. multispinosus*. This was based on the high host-parasite specificity criteria of *C. multispinosus* on *Pampus chinensis*. In terms of structural divergence, the two species was grouped into two different taxon based on the structure of the abdomen. *Caligus rotundigenitalis* is armed with an abdomen, length less than half of its cephalothorax, while the abdomen of *C. multispinosus* length is more than three quarter of its carapace (Ho *et al*., 2000). Besides that, structure-wise also declared that *C. rotundigenitalis* is different from *C. tanago* based on the structure of their 2-segmented adomen. However, this result was supported by the assumption that the shape of abdomen remains unchanged with maturity.

Misidentification incidence related to *C. chiastos* was discussed by Hayward *et al*. (2008). The species was believed to be the causative organism in the Australasian regions instead of *C. elongatus* and *C. rapax* was reported by Parker (1969), Rough (2000), Munday *et al*. (2003) and Nowak (2004). Morphology-wise, this species was simplified into an individual *Caligus* taxon based on the characteristic features of second and fourth thoracopod (Chapter 3).

Caligus rotundigenitalis is acknowledged as one of the cosmopolitan caligid species as it can be found worldwide and within a broad range of host (Ho *et al*., 2000). In Taiwan,

there were 32 fish species reported as the hosts for *C. rotundigenitalis* (Ho and Lin, 2004). Yuniar e*t al*., (2007) reported this parasite was isolated from *Mugil cephalus* from Indonesian water with the prevalence of 24.3%. Furthermore, this species is also known to parasitize *Oreochromis mossambicus* cultured in brackish water (Lin and Ho, 1993). *Caligus rotundigenitalis* was also acknowledged as a potential threat to Asian aquaculture (Ho, 2000) with adverse effects such as slow growth or mortalities in cultured fish (Lin *et al*., 1997).

As for *C. chiastos*, this species has not been reported to infect Asian seabass except marine cultured snappers (Venmathi Maran *et al*., 2008). In the present study, only one adult female of *C. chiastos* were collected from cultured fish at Bukit Tambun. This low abundance reflects that this species is very rare. Similarly, both *C. epidemicus* and *C. rotundigenitalis* have no specific host and thus, the tendency to change host is high. This species was first isolated in Australia with only one female found to infest snapper, *Chrysophrys auratus*, Forster (1801) (Roubal *et al*., 1983) and thereafter from *Plectorhynchus cinctus*, Temminck and Schlegel (1843) in Taiwan (Lin and Ho, 2003). A large number of *C. chiastos* were found to infest farmed *Thunnus maccoyii*, Castelnau (1872) in Australia (Hayward *et al*., 2008).

However, extension studies are required for the newly found species which was isolated in 2011 (Muhd Faizul *et al*. (2011). The first priority is to describe the unknown species because *C. punctatus* was reported previously to infect *L. calcarifer* in Malaysia. Based on the primary observation using dissecting microscope, the structures of the new species was dissimilar with other reported species to occur in Malaysia. It is approximately

four times longer than the length of other reported species. Therefore if we are able to proceed with SEM, it should be one of the major outputs of this present study.

The main focus of this objective is to present with clear and concise structures of the causative *Caligus* species. The major outcome of this study is generally would help the target population (farmers) to know about the pathogens which are causing trouble to the industry. Strategized by a complete pictures of the attachment hook structures, the farmers would able understand the ability of this parasite to cause injuries to their fishes. Besides, it would able to realize of how this species would be able to infect other types of host. Thus, in future, the farmers would be more careful particularly on transporting their fishes.

This is due to the caligids host finding behavior. The species tend to switch host and therefore could infect various marine organisms. Target population (farmers) able to differentiate the species and know about their present industrial enemy.

6.3 Phylogenetic analysis based on 28S rRNA

In general, the inferred phylogenetic analysis (Fig. 5.1) eminently reveals the genecology relationship between the Malaysian isolated specimens with Caligidae and Siphonostomatoida taxa retrieved from the GenBank™. Generally, the lineage among the taxon is compatible with the morphology-based classification. This is due to the separation of five well-distinguished order of Copepoda (Fig. 5.1). All of the selected orders are apparently distinctive groups of copepods which are well-separated from Hexapoda (*M. hrabei*). Calanoida appears to be the most ancient group. It forms in as a basal to the major group of copepods. It is illustrious that Poecilostomatoida is the closest relative to Siphonostomatoida (*Caligus*), followed by Harpacticoida. This arrangement is constantly been reproduces by all analysis.

Thus, in common, the phylogenetic trees presented in this study are compatible with the well-accepted morphology-based phylogeny proposed by Huys and Boxshall (1991) (Fig. 2.10). The phylogeny summarized on the formation of monophyletic clade of Mormonilloida, Harpacticoida, Poecilostomatoida, Siphonostomatoida and Monstrilloida. The Cyclopoida forms sister-group to the clade, while Calanoida and Platycoida formed in a separated group basal to the others. Our analyses however comprises with only five of the retrieved orders; Harpacticoida, Poecilostomatoida, Siphonostomatoida, Cyclopoida and Calanoida. Each order forming their own monophyletic group, which is structured similarly as the published literature.

The generated trees are also compatible with 28S rRNA copepods phylogenetic tree proposed by Tjensvoll (2006). Monophyly relationship of *Caligus*, Caligidae and Copepoda

were well-illustrated by all analyses (Figure 5.1). Most recent study on 28S rRNA analysis of *Caligus* has revealed the close relationship in between *C. chiastos* and *C. elongatus* (Nowak *et al*., 2011). Like-wise, this type of relationship was also delivered by our analysis. It shows that these two species are monophyletic with the exception that not all the taxa in Nowak *et al*. (2011) are used in this study (Fig 5.1). This study is focusing on the nearly complete 28S rRNA gene to differentiate the copepods rather than narrow down to D1-D2 region. Besides the compliment, 28S rRNA (D8-D9) analysis of Copepoda proposed by Braga *et al*. (1999) has unveiled a different set of result. Although the separation is not involving either *Caligus* or siphonostomatoids, the parsimony tree inferred by the study illustrated that Calanoida is sister group to Harpacticoida and Poeciostomatoida is the basal group. To compare, this present study simplified that Calanoida is the most primitive group of Copepoda (Figure 5.1).

An inconsistency was observed between the arrangements of *Caligus*. It is noted that the topology of both NJ and ML are compatible with the ordinary classifications. All of the isolated species are incorporated into the genus as expected. This genus later forming a sister-group relationship with *Lepeophtheirus* von Nordmann (1832). In contrast, MP analysis placed *C. epidemicus* outside of neither *Caligus* nor *Lepeophtheirus*. Moreover, the branching pattern of *C. rotundigenitalis* is also differing in MP, whereby, it shows that the species evolve from *C. curtus*. These variations might be derived from the differences in the methods used in constructing the trees. Different algorithm used in inferring the data might affect the results; MP is strategizing on the parsimony informative sites, NJ is based on distance matrix algorithm calculated from multiple sequence alignment and ML is more on statistical measures of the data set. These variations allow present study to observe the

divergences of the same set of sequences inferred by the three different phylogenetic methods. Additional studies are required to seek for the best tree algorithm.

Vast literatures have highlighted the in-stability of phylogeny of Copepoda. It is known that high degrees of diversity were observed in phylogenetic analyses of arthropoda and crustaceans (Turbeville *et al*., 1991; Giribet and Ribera, 2000; Mallatt *et al*., 2004). The uncertainty of Copepoda phylogenies is still being questioned due to the discrepancies between several published literatures, regardless whether the 18S, 28S or the mitochondrial genes. The sequences arrangement, signal and phylogenetic analysis of the 28S rRNA sequences are differ in between species.

Principally, precise phylogenetic analyses acorrespondings to the stability and accuracy of taxonomic classifications. Besides, it is often been concluded within arthropod phylogeny based on rRNA sequences, more taxa or more data might result in higher tree resolution (Spears and Abele, 1997). In this study, it was observed that the exploration of *Caligus* Müller (1785) and as well as the Copepoda are still very far from complete. As known, *Caligus* is a genus which regarded as the largest of Copepoda whereby, it is comprised of more than 250 species. However, only *C. elongatus* and *C. curtus* are appropriate to include in this study. Noticeably, there are several other taxa such *as C. warlandi*, available in the GenBank™ have to be left out since the study was exploring the whole partial sequences of Malaysian isolate *Caligus*.

CHAPTER 7

Conclusion

In conclusion, there were a total of four species of *Caligus* Müller (1785) known to parasitize cultured *L. calcarifer* in Malaysia; *C. epidemicus*, *C. rotundigenitalis* and *C. chiastos* (present study) and *C. punctatus* from (previous study). Pre-dominant infection by *C. epidemicus* was observed throughout the study; constituting up to 93.8% of the total isolations, mean abundance of 2.236 and presence at all sampling sites. No isolation of *C. punctatus* was recorded in this study. The morphological analysis using Scanning Electron Microscopy (SEM) clarified *C. epidemicus*, *C. rotundigenitalis* and *C. chiastos* into their expected ordinal (Copepoda: Siphonostomatoida: Caligidae: *Caligus*). This clarification was supported by the phylogenetic analysis based on the partial 28S ribosomal RNA. The reference sequence signalized for the incorporation of the isolated species into the monophyletic group of Copepoda, Siphonostomatoida and Caligidae.

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Caligus Müller (1785) reported to parasitize finfish in Malaysia.

Species	Host	Origin of host	Area	Killer species
C. chiastos Lin and Ho (2003)	*L. eryhtropterus* *L. johnii*	Cultured	Langkawi and Bukit Tambun	No
C. eleutheronemi Shen (1957)	*Eleutheronema tetradactylum*	Wild	Penang	No
C. epidemicus Hewitt (1971)	*L. calcarifer* *E. coioides*	Cultured	Penang	Yes
C. epinepheli Yamaguti (1936)	*Nemipterus japonicus* *Sillago sihama*	Wild	Penang	No
C. kanagurta Pillai (1961)	*Rastrelliger kanagurta*	Wild	Penang	No
C. laticaudus Shiino (1960)	*Formio niger*	Wild	Penang	No
C. longipedis Bassett-Smith (1898)	*G. specious*	Cultured	Penang	No
C. malabaricus Pillai (1961)	*Lutjanus malabaricus*	Wild	Penang	No
C. multispinosus Shen (1957)	*Pampus chinensis*	Wild	Penang	No
C. punctatus Shiino (1955)	*L. calcarifer*	Cultured	Penang	Yes
C. rotundigenitalis Yü (1933)	*L. eryhtropterus,* *E. Bleekeri* *E. fuscoguttatus*	Cultured	Penang	Yes

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SCANNING ELECTRON MICROSCOPY OF THREE SPECIES OF *CALIGUS* (COPEPODA: CALIGIDAE) PARASITIZED ON CULTURED MARINE FISH AT BUKIT TAMBUN, PENANG

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Mass mortality of fish due to infestation of C.epidemicus in Australia and Taiwan had been reported. The survey of ectoparasites on cultured fish at Bt Tambun, Penang showed three caligid, Caligus epidemicus, C. chiastos and C. rotundigenitalis parasitized the body and gill of the cultured fish. In the present study, 3 species *C. epidemicus, C. chiatos* and *C. rotundigenitalis* of the 10 species of dangerous sea lice listed were found in Malaysia. *Asian seabass shows the highest prevalence of 100% compared to 28% in white spotted snapper. SEM revealed some morphological differences in size and the shape of these three crustacean parasites. Further differences were seen in the furca, abdomen and their four-legged structure. The present study highlighted their morphology under SEM which gives us more information on these three caligid.*

Keywords: ectoparasites, Asian seabass, white spot snapper, *Caligus epidemicus, C. chiastos* and *C. rotundigenitalis*

INTRODUCTION

Infections in maricultured fish are predominantly caused by monoxenous parasites, in particular the ectoparasites. Previous studies of parasites found in marine cultured fish indicated ectoparasites such as *Benedenia* spp., *Caligus* spp and marine leech (*Zeylanicobdella arugamensis*) [1, 2 & 3]. Normally the ectoparasites were found on fish with scale drop, tail and fin rot symptoms [4]. Approximately 10 species of sea lice have been considered dangerous species on maricultured fish [5].

The survey of ectoparasites prevalen on cultured fish at Bt Tambun, Penang showed three caligid, C. *epidemicus, C. chiastos* and *C.rotundigenitalis* parasitized the body and gill of the cultured fish. In the present study, 3 species *C. epidemicus, C. chiatos* and *C. rotundigenitalis* of the 10 species of dangerous sea lice listed were found in Malaysia. Mass mortality of fish due to infestation of *Caligus epidemicus* in Australia and Taiwan had been reported [6]. Description of *Caligus epidemicus, C. chiastos* and *C. rotundigenitalis* have been reported in Malaysia cage culture systems, however no prevalence was highlighted [7]. Hence, it is important to study their morphology under SEM for more information on these three caligid.

METHODS AND MATERIALS

A total number of 10 asian seabass (*Lates calcarifer*) and 14 of white spot snapper *Lutjanus erythropterus*) from floating cages in Bukit Tambun, Penang, Malaysia (Lat. 5° 25'N; Long. 100° 19'E) were randomly sampled from different cultured periods for examining the presence of the parasites. Sources of *Caligus* spp. were obtained from the body and gill of cultured

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fish, asian seabass and white spot snapper. The *Caligus* spp. were isolated by hand and placed into glass container with 300 ml sea water before transporting to the laboratory.

Caligus spp were washed thoroughly before fixing in McDowell-Trump solution. The specimens were post-fixed in 1.0% osmium tetroxide, dehydrated through graded series of alcohol and immersed in hexamethyldisilazane (HDMS) for 10 minutes before air dried in the desiccator. Specimens were mounted on to a SEM specimen stub with a double-sided sticky tape, coated with gold, and viewed under LeoSupra 50VP Field emission SEM equipped with Oxford INCA400 energy dispersive x-ray microanalysis system at magnifications of 27x – 1.27Kx. Identification was based on morphological features [8] while the prevalence and mean intensity of parasites were recorded [9].

RESULTS AND DISCUSSION

Three species of caligids, *Caligus epidemicus, C. chiatos* and *C. rotundigenitalis* were revealed in the present study. *Caligus epidemicus* was found only in asian seabass while *C. chiatos* and *C. rotundigenitalis* were seen in white spotted snapper. Asian seabass scored the highest prevalence of 100% compared to 28% in white spotted snapper (Table 1& Fig. 1).

Table 1: Prevalence of *Caligus* sp in marine cultured fish in floating cages at Bt. Tambun, Penang.

Host	No.examined	No.infected with *Caligus* sp	Prevalence * (%)	Parasitic crustacean species
Asian seabass	10	10	100	*C.epidemicus*
White spotted snapper	14	4	28.6	*C.chiastos & C.rotundigenitalis*
Total	24	14	58.3	

**= (number of infected fish/number of fish examined) x 100%*

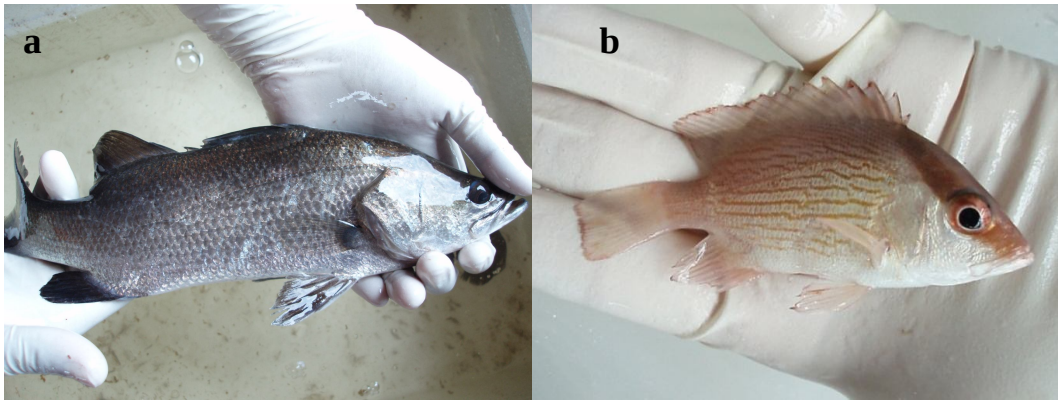

Fig. 1. The host of crustacean parasite obtained from marine cages in Bt. Tambun (a). Asian seabass (*Lates calcarifer*) and (b) white spotted snapper (*Lutjanus erypthropterus*)

SEM revealed some morphological differences in size (Table 2) and shape (Fig. 2) of these three *Caligus* spp infecting the marine cultured fish. Ten specimens of each *C. epidemicus* and *C. rotundigenitalis* while only 1 specimen of *C.chiastos* were described and measured (in micrometers, µm). Under SEM, further differences were seen in the sternal furca, abdomen (genital segment) and their four-legged structure among these three caligid (Fig. 3). Sternal furca of *C. chiastos* was proximal subquadrate box and bluntly pointed as compared with *C. rotundigenitalis* which was slightly ovate and bulging tines. Both of *C. chiatos* and *C.*

rotundigenitalis consists of two segmented small abdomen but the later was not distinct. Genital segment of *C. epidemicus* was subrectangular as compared with suborbicular in *C. chiatos*. *Caligus rotundigenitalis* seemed to have a larger four-legged structure compared to *C. chiastos* and *C. epidemicus.* *Caligus epidemicus* has a long exopodal spine which were not seen in fourth leg of *C. chiatos* and *C. rotundigenitalis*. The differences observed at the sterna furca, abdomen and the four-legged structure between these three caligids increased the basic knowledge on their morphological under light microscropy.

Table 2. Measurement of three caligid isolated from marine cultured fish in floating cages at Bt. Tambun, Penang.

	Measurement (Average length ±STD x Average wide ± STD) mm		
Species	*Caligus epidemicus*	*Caligus rotundigenitalis*	*Caligus chiastos*
Host	Asian seabass, *Lates calcarifer*	White spotted snapper, *Lutjanus erypthropterus*	White spotted snapper, *Lutjanus erypthropterus*
Total length	Male: 1.18 ± 0.05 Female: 2.94 ± 0.10	Male: 1.90 ± 0.06 Female: 3.23 ± 0.18	Male: 2.16
Cephalothorax	Male: 0.93 ± 0.09 x 0.88 ± 0.15 Female: 1.27 ± 0.06 x 1.30 ± 0.24	Male: 1.13 ± 0.18 x 0.99 ± 0.10 Female: 1.27 ± 0.10 x 1.21 ± 0.11	Male: 1.19 x 1.09
Genital Segment	Male: 0.23 ± 0.028 x 0.29 ± 0.01(anterior) 0.12 ± 0.01(posterior) Female: 0.41 ± 0.04 x 0.65 ± 0.06	Male: 0.34 ± 0.10 x 0.32 ± 0.09 Female: 0.63 ± 0.11 x 0.61 ± 0.09	Male: 5.61 x 5.73
Abdomen (excluded caudal lamella)	0.12 ± 0.07 x 0.12 ± 0.05	0.34 ± 0.04 x 0.23 ± 0.04	Male: 4.20 x 5.70

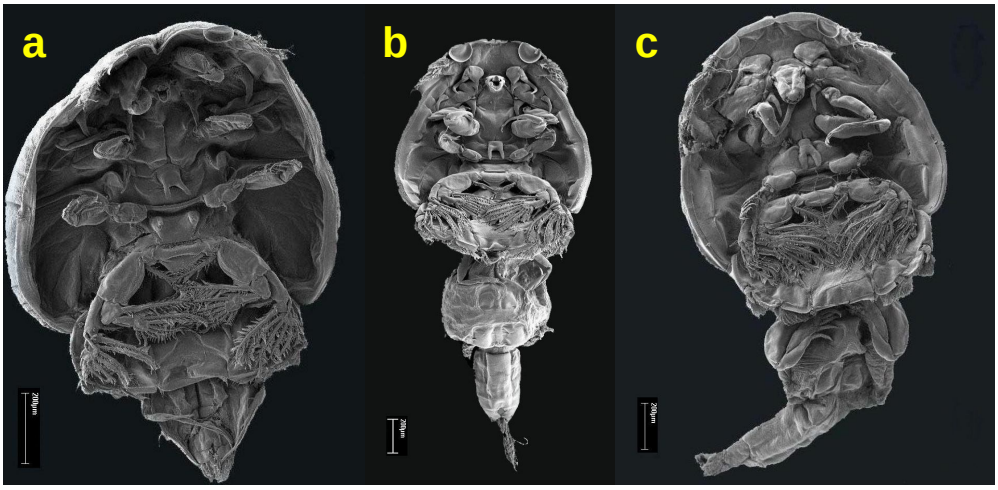

Fig. 2. Morphological characteristic between the male of *C.epidemicus* (a), *C.chiastos* (b) and *C. rotundigenitalis* (c) under SEM

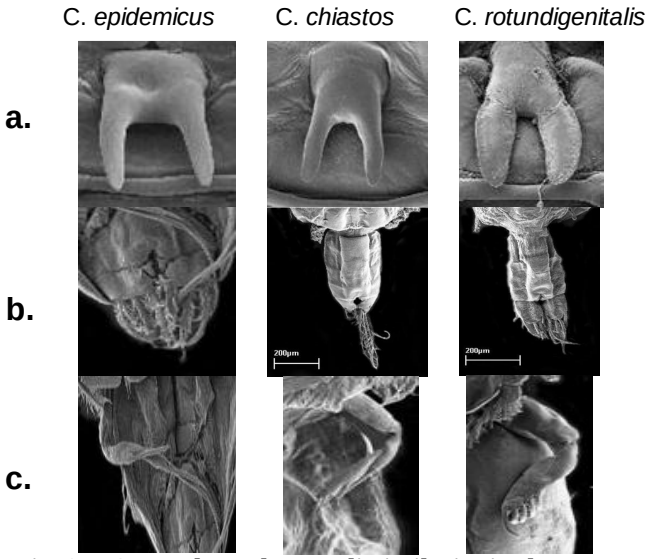

Fig. 3. SEM showed great dissimilarity in three organs such as furca (a), abdomen (b) and the four-legged (c) among the three caligid.

CONCLUSION

SEM study showed great dissimilarity in the three main organs of fuca, genital segment and the four-legged structure among the three caligid, C. *epidemicus, C. chiastos* and *C.rotundigenitalis* which parasitized the body and gill of the cultured fish. Hence, their morphological differences as revealed under SEM help in the further identification of these three caligid.

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Caligidae infestation in Asian seabass, *Lates calcarifer*, Bloch 1790 cultured at different salinity in Malaysia

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ABSTRACT

The Asian seabass is euryhaline, therefore it is interesting to describe the infestation and survival of caligids at varying salinity on the host. In this study, two different brackish water culture systems with monoculture and polyculture practices were investigated for the occurrence of *Caligus* spp. on *Lates calcarifer*. Polyculture practices mainly consisted of snapper (*Lutjanus* spp.), grouper (*Epinephelus* spp.) and seabass (*L. calcarifer*), while the monoculture was stocked with only seabass. A total of 777 *Caligus* spp. specimens were isolated from the sampling in 2009, consisting of three species; *Caligus chiastos*, *Caligus epidemicus* and *Caligus rotundigenitalis*. In 2011, the total specimen was increased to 3110 and two additional species were found; *Caligus punctatus* and one unknown species (*Caligus* sp.). A 98.6% of the total examination was represented by *C. epidemicus*. Constant presence of *C. epidemicus* was observed throughout the study, regardless the differences in between culturing practices and systems. This species was able to survive within wide salinity range, from 5 to 28 ppt. The other isolated species (*C. chiastos*, *C. punctatus*, *C. rotundigenitalis* and *Caligus* sp.) were only found infesting in polyculture cages with the salinity ranging from 25 to 28 ppt. Despite accounts for less than 2% of the total specimens, these species may able to produce a challenge for *L. calcarifer* polyculture farming activity due to their capability for host switching. The present study revealed the potential risk for cross-species transmission in polyculture practices.

1. Introduction

Asian seabass (*Lates calcarifer*) is commercially one of the most important cultured finfish within the Indo-Pacific region (Cheong, 1989). Malaysia like in most countries of South East Asia, *L. calcarifer* is cultured in fresh water, brackish water and also marine systems, in either earthen ponds or cages (Kungvankij et al., 1986). There is high demand on this species as it can be produced throughout the year at a reasonable price. Thus, higher productivity is desired by increasing the stocking which might facilitates parasitic transmission (Kabata, 1985). It is reported that the Asian aquaculture sector including Malaysia are facing severe challenges due to parasitic copepod invasion. Such parasites are known to possess a single host-life cycle and reproduces rapidly. This characteristic is an advantage for them to alter and proliferating in aquaculture since the culturing area condensed with their targeted host. Basically the information on these parasites is still rarely documented in Asian countries including Malaysia.

The first report on *Caligus* parasites on Malaysian fishes was in 1984 (Leong, 1984). There was a big gap in between that until Venmathi Maran et al. (2009) reported five species of *Caligus* that have been identified to cause problems to the aquaculture sector in Malaysia. They were

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Fig. 1. Location of sampling sites. Abbreviations: A, Santubong; B, Sematan; C, Bkt. Tambun; D, Semarahan; E, Pendas and F, Gelang Patah.

Caligus chiastos, *Caligus epidemicus*, *Caligus rotundigenitalis* and *Caligus punctatus*. Of these, only *C. epidemicus* and *C. punctatus* infest *L. calcarifer*. Kua and Faizul (2010) stated that *C. epidemicus*, *C. chiastos* and *C. rotundigenitalis* were commonly found in cultured marine fish at Bukit Tambun, Penang. Thus, the present study was carried out to determine the occurrence and adaptability of caligid infection on seabass cultured at varying salinity in Malaysia. Besides, a comparison of this parasite's invasion between culturing systems and practices had been carried out. This is vital as seabass is able to adapt into a wide range of water salinities.

2. Material and methods

2.1. Sample collection and water quality (salinity parameter)

Seabass cultured from four states, namely Sarawak (Santubong and Semantan), Johor (Gelang Patah and Pendas), Penang (Bukit Tambun) and Kelantan (Semarahan) were obtained (Fig. 1). Sample size ranging between 5 and 70 seabass were collected randomly using fish net at floating cages and fishing rod at pond. The randomly selected fishes were not restricted to certain a size, treatment and their origin. The water salinity of each sampling site was determined using a multiprobe water quality instrument (YSI 556 MPS, USA). Four readings were taken at each site.

2.2. Collections of parasites

The fish was treated with freshwater for 15 min to collect the parasites and equipped with aeration supplies. After 15 min, the fish was returned back to the cages or pond. The freshwater was examined for the presence of *Caligus*. The dislodged parasites were collected and counted in order to determine the prevalence, mean abundance and mean intensity. The parasites were preserved in 70% ethanol for further identification.

2.3. Identification of parasites

The parasites were stained using Alum-carmine for identification. The specimens were observed with a dissecting microscope (Leica Zoom 2000 and DM 5000) at eye piece magnifications of 50–400×. Identification was based on the morphological features according to Hewitt (1971), Lin and Ho (2003) and Venmathi Maran et al. (2009). Caligids were sorted and photographed. The prevalence, mean abundance and mean intensity were calculated based on Bush et al. (1997). Some samples were sent to Prof. Ju Shey Ho from Department of Biological Sciences, California State University, CA for confirmation in identification.

Table 1
Information on the sampling sites was conducted in 2009 and 2011.

Type of culture	Location	Geographical location		Culture system	2009		2011	
		Latitude	Longitude		Sampling date	Mean salinity (ppt), $n=4$	Sampling date	Mean salinity (ppt), $n=4$
Monoculture	Gelang Patah	1.436027	103.578415	Earthen pond	17-November	14.30 ± 0.02	19-April	18.63 ± 0.03
	Semantan	1.790995	109.790754	Floating cages	09-October	24.52 ± 0.02	07-June	23.17 ± 0.02
Polyculture	Bkt Tambun	5.281964	100.396194	Floating cages	15-October	28.30 ± 0.02	–	–
	Semarahan	5.864468	102.496610	Floating cages	11-November	5.48 ± 0.02	05-April	16.41 ± 0.33
	Pendas	1.380785	103.642732	Floating cages	17-November	28.49 ± 0.02	19-April	27.59 ± 0.13
	Santubong	1.413165	110.202327	Floating cages	08-October	25.43 ± 0.05	6-June	27.28 ± 0.02

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Table 2
The occurrence of *Caligus* spp. at different salinities of seven culturing *L. calcarifer* areas in Malaysia.

	Location	Year	No. of fish	Mean size of fish (range), cm	No. of isolations (prevalence, %) (mean abundance) (mean intensity)				
					C. chiastos	*C. epidemicus*	*C. rotundigenitalis*	*C. punctatus*	*C.* sp.
Monoculture	Glg Patah	2009	5	37 (35–39)	NI	138 (100) (27.6) (27.6)	NI	NI	NI
		2011	30	18 (17–21)	NI	2386 (100) (79.53) (79.53)	NI	NI	NI
	Semantan	2009	70	21 (19–25)	NI	17 (–) (0.24) (–)	NI	NI	NI
		2011	24	22 (20–30)	NI	3 (12.5) (0.13) (1)	NI	NI	NI
Polyculture	Bkt Tambun	2009	70	25 (21–30)	1 (1.4) (0.01) (1)	11 (11.43) (0.15) (1.38)	4 (2.86) (0.05) (2)	NI	NI
		2011	–	–	–	–	–	–	–
	Pendas	2009	70	20 (15–25)	NI	471 (100) (6.73) (6.73)	43 (–) (6.10) (–)	NI	NI
		2011	30	28 (25–30)	3 (10) (0.1) (1)	407 (100) (13.57) (13.57)	1 (3.33) (0.03) (1)	1 (3.33) (0.03) (1)	NI
	Semarahan	2009	70	24 (18–26)	NI	81 (100) (1.15) (1.15)	NI	NI	NI
		2011	30	25 (15–30)	NI	261 (100) (8.7) (8.7)	NI	NI	NI
	Santubong	2009	41	26 (15–30)	NI	11 (–) (0.27)(–)	NI	NI	NI
		2011	30	31 (28–33)	1 (3.33) (0.03) (1)	35 (66.67) (1.17) (1.75)	NI	NI	1 (3.33) (0.03) (1)

NI, no infection was found.

3. Results

Two different brackish water culturing systems (earthen pond and floating cage) with monoculture and polyculture practices were investigated for the occurrence of *Caligus* spp. on *L. calcarifer*. Polyculture practices comprises mainly snapper (*Lutjanus* spp.), grouper (*Ephinephelus* spp.) and Asian seabass (*L. calcarifer*), while monoculture only consists with Asian seabass. Sample size collected varied according to the stock available and tolerance of farmers. Each sampling site differs in salinity level, ranging from 5 to 28 ppt (Table 1).

A total of 3876 specimens of caligids were isolated throughout the study, consisting with five different species; *C. chiastos*, *C. epidemicus*, *C. rotundigenitalis*, *C. punctatus* and one unknown species. Among these, *C. epidemicus* was the only species consistently found in all culturing areas, constituting for up to 98.6% of the total isolates (Table 2). Its presence was recorded within a wide salinity range; 5–28 ppt. The numbers of the other four species were relatively very small and restricted only in polyculture floating cages. Their presences were observed within a narrow salinity range; 25–28 ppt.

The isolated caligids consist of different life stages; adult, pre-adult, chalimus and pre-copulation. However, these data were only drawn from the sampling in 2011 (Table 3). Brackish water earthen pond in Gelang Patah shows 100% of infections by all stages.

4. Discussion

The first disclosure of the parasitism of *Caligus* spp. on *L. calcarifer* in Malaysia begins with *C. epidemicus* in 2003, followed by *C. punctatus* three years later (Venmathi Maran et al., 2009). This present study revealed three additional species, *C. chiastos* and *C. rotundigenitalis* from the sampling in 2009 and *Caligus* spp. in latest observation in 2011. This addition accounts for a total of five species associated to the infection onto the *L. calcarifer*. Among the species, *C. epidemicus* was the only species consistently been obtained from the host throughout the timeline (2003 until 2011), in both culturing practices (mono and polyculture) and at varying range of water salinities.

This present study also revealed that *C. epidemicus* have the largest *Caligus* population infecting cultured *L. calcarifer* in Malaysia. This species accounts for 98.6% and infecting nearly 60% of the total examined host. The adaptability of this caligids to wider water salinity range was observed from 5 to 28 ppt. This result supports the report on the species of *Caligus* spp. found at salinity ranging from 4 to 28 ppt in Australia (Hewitt, 1971). A large number of chalimus stage reflecting the fact that the caligid are able to tolerate at low salinity and invade brackish water fishes (Lin and Ho, 1993). On the other hand, it also indicated that the population growths activity of caligid which can cause more infection to the cultured fish. *C. epidemicus* was known to infect various cultured finfish in Philippines, cultured grouper in Vietnam, mullet and porgies in Taiwan and also snapper cultured in Malaysia (Leong and Wong, 1988; Ho et al., 2004; Vo et al., 2008; Kua and Faizul, 2010). Moreover, the infected hosts were not only restricted to fish

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Table 3
The occurrence of different life stages of *Caligus* spp. at all sampling sites drawn from the sampling in 2011.

Locations	No. of specimen (Prevalence, %)				
	Adult female	Adult male	Pre-adult	Chalimus	Precopula position
Semarahan	151 (100)	71 (100)	8 (16.6)	19 (33.3)	6 (20)
Gelang Patah	903 (100)	944 (100)	48 (100)	171 (100)	135 (100)
Pendas	226 (100)	121 (100)	8 (26.67)	38 (76.67)	12 (26.67)
Santubong	29 (66.67)	6 (16.67)	–	1 (3.33)	1 (3.33)
Semantan	2 (8.33)	1 (4.16)	–	–	–

species, the tiger prawn in Thailand was also vulnerable (Ruangpan and Kabata, 1984). Thus, due to such existence, this species is regarded as killer species for fish farming activity in Asian continents (Venmathi Maran et al., 2009).

Despite accounting for less than 2% of the population size, the presences of the other four species may trigger for a potential threat to the industry. This is due to the fact that *C. chiastos*, *C. rotundigenitalis* and *C. punctatus* are cosmopolitan species. Thus, they are known for the ability to adapt and proliferate onto host switching. Currently, *C. chiastos* and *C. rotundigenitalis* have not been reported to infect *L. calcarifer* but closely been associated with the others infection in cultured snapper or grouper and several other species such as *Plectorhynchus cinctus* and *Thunnus maccoyii* for *C. chiastos* and *Mugil cephalus* and *Oreochromis mossambicus* for *C. rotundigenitalis* (Roubal et al., 1983; Lin and Ho, 1993, 2003; Yuniar et al., 2007; Hayward et al., 2008; Venmathi Maran et al., 2009). Microscopic observation of the fifth species (*Caligus* spp.) revealed that it was approximately four times longer than the first four species. It shows the typical caligids tagmatization, by possessing cephalothorax, fourth leg bearing segment, genital segment and abdomen. The species was well-equipped with lunules at its frontal plate, suggesting it is a *Caligus* and not *Lepeophtheirus*. A further descriptions and species identification was still in progress.

This study also unveiled the vulnerability of *L. calcarifer* cultured in polyculture activity to the infections by the other species (Table 2). More species were discovered as compared to the opposite practice. This situation might derived by a close interaction in between the cultured stocks, while monoculture activity is able to minimize such contact. However, this result should cautiously take since the presences of these caligids are directly affected by the farm managements. Further studies are required in order to justify this assumption. Besides, the floating net cages also allow the interaction in between the cultured stock with the wild-origin fish. This situation is one of the important areas that need to be highlighted, whether the parasitic organisms were either transmitted into the farm or out into the wild.

5. Conclusion

Lates calcarifer culturing industry in Malaysia were found infected by five species of *Caligus*; *C. chiastos*, *C. epidemicus*, *C. rotundigenitalis*, *C. punctatus* and one unknown species (*Caligus* spp.). This study also illustrates the constant infection caused by *C. epidemicus* onto the host, regardless the differences in salinity, geographical area, practice and timeline. Despite accounts for low percentages, the four other species may able to produce a challenge for the industry due to their capability for host switching, particularly at 24–28 ppt.

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Caligus Müller, 1785 parasitizing cultured *Lates calcarifer* Bloch, 1790 in Malaysia

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Abstract: Present study unveils the parasitism of the three *Caligus* Müller (1785) species in cultured *Lates calcarifer* Bloch (1790) in Malaysia. The divergences in between the species was investigate through three perspectives: the presence, morphological and phylogenetic. Scanning electron microscopy conclusively reveals the species as *C. chiastos*, *C. epidemicus* and *C. rotundigenitalis*. Incessant infection by *C. epidemicus* was observed throughout the study; more than 90% of the total isolated specimens showed presences at all observed states. Followed by the infection by *C. rotundigenitalis*, it covered approximately 6% of the total specimens isolated from Johor and Penang. One individual of *C. chiastos* was isolated in Penang. Molecular phylogenetic analysis of partial 28S rRNA summarized the three species into monophyletic group of Caligidae. *C. chiastos* forming monophyletic relationship with *C. elongates*, *C. rotundigenitalis* evolves from the group and finally *C. epidemicus* tend to be the most primitive. So far there is no report on the parasitic invasion by *C. chiastos* and *C. rotundigenitalis* onto *L. calcarifer*. This result would enhance the knowledge regarding on the parasitic infection in aquaculture in Malaysia.

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Morphology and phylogenetic study of *Caligus* Müller (1785) parasitizing cultured Asian seabass, *Lates calcarifer* Bloch (1790) in Malaysia

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ABSTRACT

Lates calcarifer Bloch (1790) is the most marketable cultured finfish in Malaysia. This profitable host species were found to be infected by five low-host specificity species of *Caligus* Müller (1785). Present study was strategized to unveils the divergences between three of the known-species through two biological perspectives; morphological and phylogenetic. Morphological study under scanning electron microscopy (SEM) conclusively reveals the species as *C. chiastos*, *C. epidemicus* and *C. rotundigenitalis*. This study discussed more on the unique structures of their tagmata; cephalothorax, fourth thoracopod, genital segment and abdomen. Molecular phylogenetic analysis of partial 28S rRNA summarized the three species into monophyletic group of Caligidae. *Caligus chiastos* forming monophyletic relationship with *C. elongatus*, *C. rotundigenitalis* evolves from the group while *C. epidemicus* tend to be the most primitive species. The parasitic invasion of *C. chiastos* and *C. rotundigenitalis* onto *L. calcarifer* is very new to the Malaysian aquaculture industry. The upshot of this present study is a concrete evaluation through morphological and molecular identification of the species occurred in Malaysia. Such information would be vital since there were reported misidentification cases in Malaysia. This would also enhance the knowledge regarding on the parasitic infection in aquaculture activity in Malaysia particularly on the evolutionary perspectives.

Keywords: *Caligus*; *L. calcarifer*; SEM; 28S rRNA