Molecular Phylogeny of Penaeoidea, Penaeidae and *Penaeus sensu lato*

MA, Ka Yan

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Prof CHU Ka Hou (Supervisor)

Prof WONG Chong Kim (Chairman)

Prof ANG Put Jr. (Committee Member)

Prof CHAN Tin Yam (External Examiner)

Declaration

I declare that this thesis represents my own works and that it has not been previously included in a thesis, dissertation or degree, diploma or other qualification.

Signed .....

Abstract

Marine shrimps of superfamily Penaeoidea constitute a diverse group of crustaceans. Despite their ecological and commercial significance, few attempts have been made to clarify the phylogeny at the superfamily, family and genus levels and these attempts, using either morphological and molecular data, failed to give convincing resolutions to the issues. In this study, the phylogeny of Penaeoidea, Penaeidae and *Penaeus sensu lato* were investigated using new molecular data.

Attention to the phylogeny of the Penaeoidea is raised since studies using mitochondrial markers have challenged the monophyly and classification of the penaeoid families. In the present study, totally 1152 bp DNA sequences from two nuclear protein-coding genes, phosphoenolpyruvate carboxykinase (PEPCK) and sodium-potassium ATPase alpha subunit (NaK), were determined from 45 penaeoid shrimps. Phylogenetic analyses using maximum likelihood and Bayesian approaches strongly support the monophyly of Solenoceridae, Aristeidae, Benthesicymidae and Sicyoniidae. Yet the last family is nested within Penaeidae, making the latter paraphyletic. Penaeoidea comprises two lineages: the former three families in one while the latter two in another. The diversification of these lineages may be related to bathymetry. The penaeid-like lineage diversified in the Triassic, earlier than the aristeid-like lineage with an origin in the Jurassic. Taxonomic revisions within Penaeoidea are also proposed for further investigation. Due to the paraphyly of Penaeidae and the low genetic divergence between the benthesicymids and the aristeids, the taxonomic status of these families has to be reconsidered.

While Penaeidae is the most species-rich family in Penaeoidea, the phylogenetic relationships among its genera are poorly understood. Previous studies based on mitochondrial 16S rRNA sequences supported the three-tribe scheme proposed by Burkenroad (1983) in general. Analyzing NaK and PEPCK sequences from 16 genera of penaeids yielded results that basically corroborate this allegation. The tribe Penaeini occupies the basal position in this family, and Parapenaeini is sister to Trachypenaeini which clusters with the sicyoniids. High genetic divergences are found among the three tribes, which are almost comparable to interfamily level in Penaeoidea (>7%). As Penaeidae is now shown to be paraphyletic, raising the tribes to family level would be a reasonable proposal.

The most controversial issue in the phylogeny of Penaeoidea is the systematic status of *Penaeus s.l.* Since Pérez Farfante and Kensley (1997) splitted the previously defined *Penaeus* into six genera, much debate has been drawn on this new classification scheme. As a natural classification scheme should reflect the evolutionary relationships among organisms, it is essential to scrutinize the phylogeny of *Penaeus s.l.* in order to settle the controversy. This study examined a total of 2410 bp sequences from three nuclear protein-genes (PEPCK, NaK and enolase) together with mitochondrial 16S and 12S rRNA gene sequences of 15 *Penaeus* shrimps. Phylogenetic analyses reveal that *Penaeus s.l.* is monophyletic and contains two lineages (*Penaeus sensu stricto* + *Fenneropenaeus* + *Litopenaeus* + *Farfantepenaeus* and *Melicertus* + *Marsupenaeus*). The genetic divergence between the two lineages is high and comparable to that among other penaeid genera.

Penaeus s.s and *Melicertus* are found to be paraphyletic and hence are not natural groupings. The old classification scheme (a single genus *Penaeus*) is found more appropriate. The present study also suggests that the ancestors of *Penaeus s.l.* might have emerged in the northwest Tethys Sea during the late Jurassic. In the Cretaceous they might have either colonized both westward to the Atlantic and eastward to the present Indian Ocean, or diversified predominantly eastward to achieve the global distribution nowadays.

In conclusion, this study has scrutinized the evolutionary history of superfamily Penaeoidea, family Penaeidae and genus *Penaeus s.l.* with high resolutions. This has provided new insights to the mode of speciation and has presented robust evidences for taxonomic revisions of these shrimps.

摘要

對蝦上科的海蝦是一群多樣性很高的甲殼類動物。雖然牠們在生態及經濟上都有著相當的重要性，但只有很少的研究試圖去闡明牠們的系統發生，而且無論這些研究是使用形態上還是分子的數據，都無法提供一個確切的定論。是次研究利用了新的分子數據來研究對蝦上科 (Penaeoidea)、對蝦科(Penaeidae)與及廣義對蝦屬(*Penaeus sensu lato*)的系統發生。

早期粒線體標記研究顯示管鞭蝦科(Solenoceridae)包括在對蝦科內，這個結果對對蝦科之單系及現行的分類產生了質疑，增加了人們對對蝦上科的系統發生的關注。是次研究對 45 種對蝦的磷酸烯醇式丙酮酸羧激酶(PEPCK)和鈉鉀幫浦第一個亞單位(NaK)這兩個細胞核基因，合共 1152 個鹼基對的序列進行了測定。基於最大似然法和貝葉斯方式的系統分析強烈地支持管鞭蝦科、鬚蝦科(Aristeidae)及深對蝦科 (Benthesicymidae)爲單系，但是發現單肢蝦科(Sicyoniidae)處於對蝦科內，顯示後者爲並系群。對蝦上科由兩個支系組成：前三個科構成一個支系而後兩者在另一個支系中。這些支系的多樣化可能與海洋深度有關，並且估計發生玄於二疊紀，對蝦類的支系於三疊紀多樣化，較源於侏羅紀鬚蝦類的支系爲早。此外，是次研究亦提出了針對對蝦上科的分類修訂：由於對蝦科是並系群，而深對蝦與鬚蝦之間只有很少的遺傳差異，牠們的等級需要重新考慮。

雖然對蝦科是對蝦上科中物種最多的一科，可是我們對牠們屬之間的系統發育關係所知極之少，之前基於粒線體 16S rRNA 基因序列的研究大體而言支持 Burkenroad (1983) 提出的三個族群系統，分析了 16 個對蝦科中的屬之 NaK

和 PEPCK 基因序列所得到的結果基本上與這一主張吻合，顯示所有的三個族群都是單系群。對蝦族(Penaeini)佔據這科中的最基部位置，鷹爪對蝦族(Trachypenaeini)和單肢蝦組成一個組，而牠們是側對蝦族(Parapenaeini)的姐妹群。三個亞科之間發現相當高的遺傳分化，可以比得上對蝦上科內科與科之間的差異(>7%)，由於現在證明對蝦科是一個並系群，所以三個族群被昇至科的分類等級將會是一個合理的提案。

對蝦上科的系統發育中最備受爭議的是廣義對蝦屬的分類狀況，自從 Pérez Farfante 和 Kensley (1997)把之前界定的對蝦屬分爲六個屬之後，引發了許多圍繞著應否運用這一套新分類系統的爭論。但是一個自然分類應該是能夠反映生物之間的進化關係，所以必須詳細研究廣義對蝦屬的系統發生以解決爭議。是次研究測定了 15 種對蝦屬的蝦之三個細胞核蛋白編碼基因(PEPCK、NaK 及烯醇化酶 enolase)與及粒線體 16S rRNA 和 12S rRNA 的基因片段序列，合共 2410 個鹼基對，系統發生分析顯示廣義對蝦屬是單系群以及包含二個支系(狹義對蝦屬 *Penaeus sensu stricto* + 明對蝦屬 *Fenneropenaeus* + 濱對蝦屬 *Litopenaeus* + 美對蝦屬 *Farfantepenaeus* 與及溝對蝦屬 *Melicertus* + 囊對蝦屬 *Marsupenaeus*)，這兩個支系之間存在高度的遺傳分化，可以和其他對蝦的屬之間的差異比擬。狹義對蝦屬和溝對蝦屬被發現爲並系群所以，牠們並不是自然編組。以此研究的結果來看，舊的分類方法(一個統一的對蝦屬)比較合宜。系統發生分析和分歧時間估計結果，對於這些物種的起源和多樣化發生的時間和地點都提供了新的理解，廣義對蝦屬的祖先可能在晚侏羅紀出現於特提斯海之西北，牠們可能在白堊紀同時往西移居到大西洋及往東至現今的印度洋，或者主要向西多樣化以達到今日的全球性分佈。

總括而言，這次研究詳細探討了對蝦上科、對蝦科與及廣義對蝦屬的進化關係並獲得清晰的解答，這對物種形成的方式提供了新的理解，同時爲這些蝦的分類修訂提出了明確的證據。

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Chapter 1

Introduction

Study of the gene at the most fundamental level will soon tell us more about the phylogenetic relationships of organisms than we have managed to learn in all the 173 years since Lamarck.

R. K. Selander (1982)

1.1 Molecular phylogenetics

Our understanding of the phylogeny of organisms from the smallest bacteria to the largest cetaceans has expanded tremendously in the past two decades, thanks to the advances in molecular biology that allow rapid accumulation of DNA sequences – "the essences of the organism" (Zuckerkandl and Pauling, 1965) that document its evolutionary history. Modern phylogenetics is almost synonymous to molecular phylogenetics, a field that attempts to reconstruct phylogeny by delineating pattern of change in molecules (especially DNA sequences of various genes) between different organisms. As there are growing concerns on the traditional morphological approach to phylogeny that analyses are often hampered by limited available characters and the difficulties to define synapomorphic traits (Avise, 2004), the molecular approach has the advantages attributable to the fact that genes do not only reveal but also engender evolution, and that there are over millions of nucleotide base pairs in a eukaryotic genome which offer a much bigger potential pool of characters than those from the morphology of an organism.

Despite the effectiveness and ease of using molecules to infer phylogeny, this by no means implies that molecular approach is ultimate, superior solution to all phylogenetic issues. Instead, concordance among independent data sets acts as a principal measure of the robustness of phylogenetics hypotheses (e.g., Penny and Hendy, 1986; Miyamoto and Cracraft, 1991; Hillis, 1995; Miyamoto and Fitch, 1995). When phylogenies inferred from morphology and molecules contrast, there are uncertainties in the proposed phylogenetics hypotheses and further studies are required.

The penaeoid shrimps represent an example of which morphological and molecular phylogenies do not concur. Due to their high economical values and because many of these shrimps have been the target of scientific researches, the ambiguity in their phylogeny would have profound effects. In this chapter I will introduce the controversies in Penaeoidea phylogeny – from superfamily to genus level, and I will discuss the suitability of different molecular markers for a better phylogenetic reconstruction of the penaeoids.

1.2 Phylogeny of the penaeoid shrimps

The penaeoid shrimps (superfamily Penaeoidea) constitute a diverse group of marine decapods with over 400 species. Globally distributed, and inhabiting both shallow-waters and abyssal zones below 5000 m, they occupy different trophic levels of the food chain at various water depths in the ocean (Pérez Farfante and Kensley, 1997). This group includes most marine shrimps of high economic value and accounts for

over one third of the annual wild crustacean catch (FAO, 2008). It is surprising that that no consensus on the phylogeny of Penaeoidea, Penaeidae and *Penaeus sensu lato* has not been reached on a firm basis and put an end to the uncertainties in their taxonomy.

1.2.1 Interfamilial relationships of Penaeoidea

Penaeoidea is commonly considered to have four families, namely Aristeidae, Solenoceridae, Penaeidae and Sicyoniidae (e.g. Holthuis, 1980; Liu and Zhong, 1986; Yu and Chan, 1986; Dall et al., 1990; Hayashi, 1992; Chan, 1998). However, the most recent classification scheme lists five families in Penaeoidea by adding the family Benthesicymidae (Pérez Farfante and Kensley, 1997; Martin and Davis, 2001). Benthesicymidae was traditionally regarded as a subgroup (i.e. series, tribe or subfamily) of Aristeidae and the suggestion that it should be ranked as a family, first made by Crosnier in 1985, went unheeded until recently. As for the other four families, Sicyoniidae is commonly believed to be close to Penaeidae while Solenoceridae is allied with Aristeidae. Such a subdivision of the superfamily coincides with the distinct adult habitat choices of the families, as the penaeids and sicyoniids usually inhabit littoral waters while the aristeids and solenocerids are mostly deep-sea species (Burkenroad, 1934, 1936; Pérez Farfante, 1977; Dall et al., 1990). However, detailed discussions of the phylogenetic relationships amongst the penaeoid families and genera have been limited, and only two comprehensive schemes have been proposed, by Kubo (1949) and Burkenroad (1983). Kubo's (1949, fig. 1.1a) scheme, although deduced from a very complicated set of characters, was based on rather limited genera. He proposed that Sicyoniidae (as Eusicyoninae) was

the most primitive while Penaeidae (as Penaeinae) was the most advanced group, with Solenoceridae (as Solenocerinae) being intermediate between Aristeidae (as Aristaeinae) and Penaeidae. Burkenroad's scheme (1983, fig. 1.1b) was more complete. Although Burkenroad (1983) only considered three genera in Solenoceridae (as Solenoceinae), Pérez Farfante (1977) elaborated the phylogenetic relationships of the then established seven solenocerid genera based mostly on Burkenroad's (1936) earlier groupings. Several genera later discovered or split from the existing genera can be readily incorporated into Burkenroad's (1983) scheme (i.e., those genera in brackets in fig. 1.1b). Burkenroad (1983) considered that Solenoceridae was the most primitive group based on fossil record (even perhaps the ancestor of Penaeoidea and sergestoidea, see also Burkenroad, 1963) and Sicyoniidae (as Sicyoninae) the most advanced.

The above morphologically-inferred phylogenies were challenged by recent phylogenetic studies with noticeably contrasting conclusions. Analyses of spermatozoa ultrastructure suggest a close relationship between Penaeidae and Solenoceridae, both with spiked acrosome and simple subacrosomal region (Scelzo and Medina, 2004; Medina et al., 2006a, b; see fig. 1.1c). In common with these two families, Sicyoniidae also has spiked spermatozoa but differs by having an elaborated subacrosomal region. Aristeidae is placed in a basal position because the spikeless spermatozoa is regarded by Scelzo and Medina (2004) as an ancestral character shared by the sergestoid shrimps (the sister superfamily of Penaeoidea). However, owing to the limited data available on spermatozoa ultrastructure and the

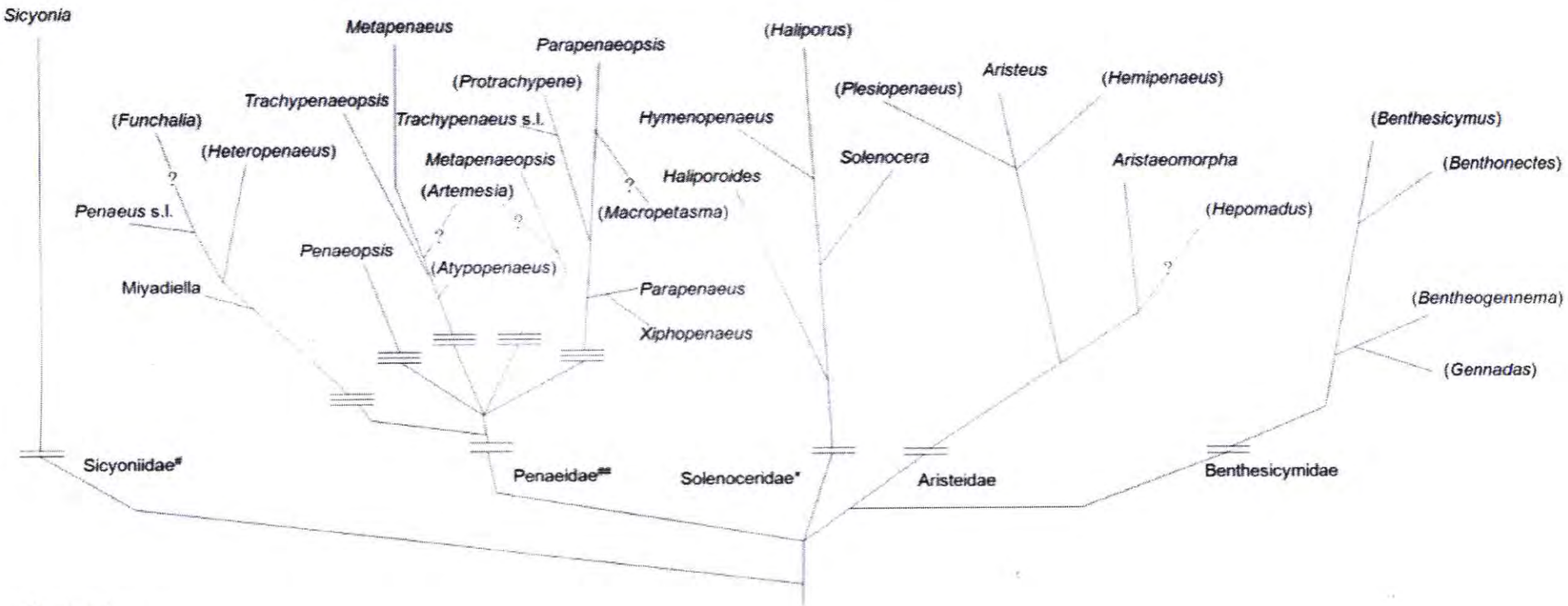
Sicyonia
Metapenaeus
Parapenaeopsis
(Haliporus)
(Protrachypene)
Aristeus
(Hemipenaeus)
(Plesiopenaeus)
Trachypenaeopsis
(Funchalia)
Trachypenaeus s.l.
Hymenopenaeus
(Benthesicymus)
(Heteropenaeus)
Metapenaeopsis
Solenocera
Aristaeomorpha
(Benthonectes)
(Artemesia)
Haliporoides
Penaeus s.l.
(Macropetasma)
(Hepomadus)
Penaeopsis
(Atypopenaeus)
Miyadiella
Parapenaeus
(Bentheogennema)
Xiphopenaeus
(Gennadas)
Sicyoniidae#
Penaeidae##
Solenoceridae*
Aristeidae
Benthesicymidae

Figure 1a

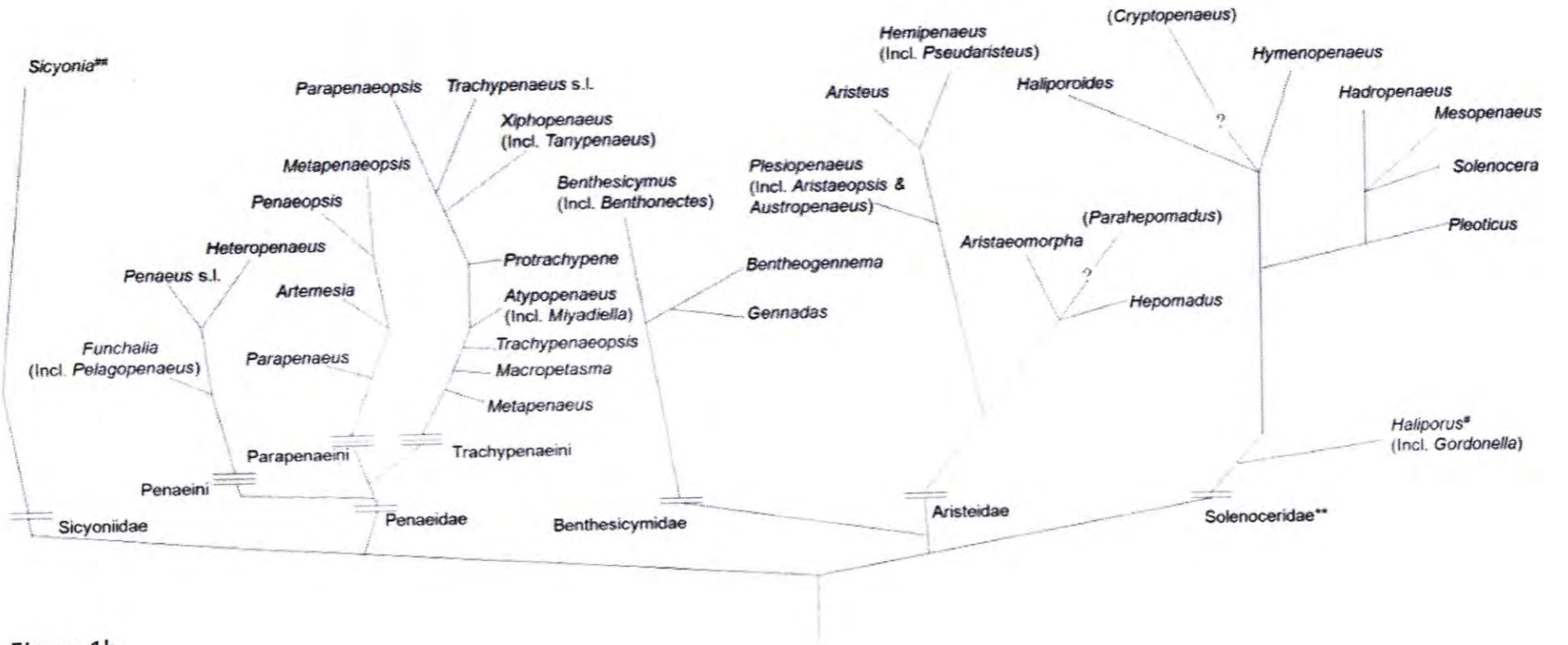
Sicyonia##
Parapenaeopsis
Trachypenaeus s.l.
Xiphopenaeus
(Incl. Tanypenaeus)
Metapenaeopsis
Penaeopsis
Heteropenaeus
Penaeus s.l.
Artemesia
Funchalia
(Incl. Pelagopenaeus)
Parapenaeus
Protrachypene
Atypopenaeus
(Incl. Miyadiella)
Trachypenaeopsis
Macropetasma
Metapenaeus
Benthesicymus
(Incl. Benthonectes)
Plesiopenaeus
(Incl. Aristaeopsis &
Austropenaeus)
Bentheogennema
Gennadas
Hemipenaeus
(Incl. Pseudaristeus)
Aristeus
Aristaeomorpha
(Parahepomadus)
Hepomadus
(Cryptopenaeus)
Haliporoides
Hymenopenaeus
Hadropenaeus
Mesopenaeus
Solenocera
Pleoticus
Haliporus#
(Incl. Gordonella)
Parapenaeini
Trachypenaeini
Penaeini
Sicyoniidae
Penaeidae
Benthesicymidae
Aristeidae
Solenoceridae**

Figure 1b

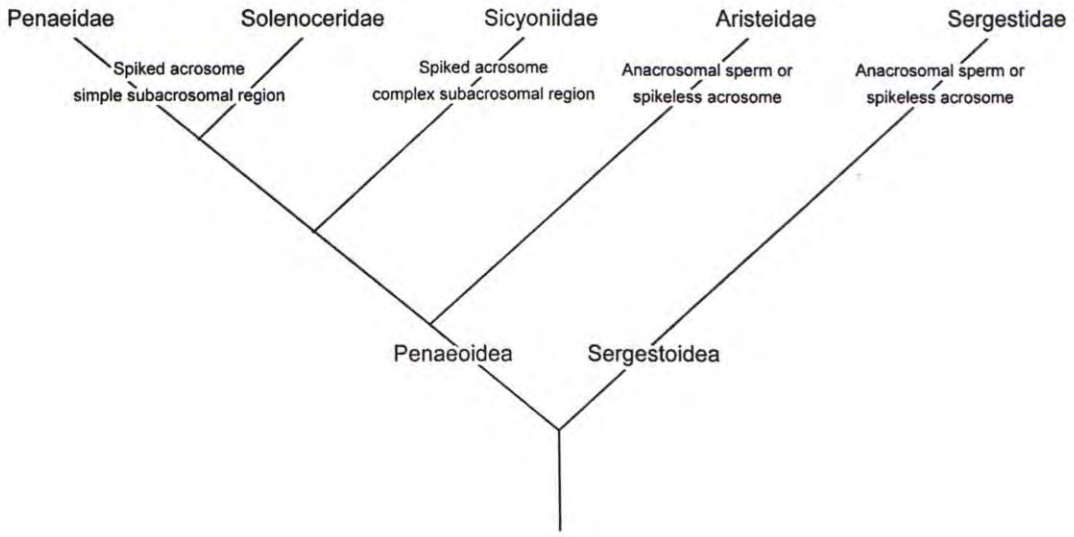

Figure 1c

Figure 1.1. Morphological phylogeny of the penaeoid genera proposed by (a) Kubo, 1949, reconstructed from text (genera in brackets were not fully analyzed), * considered as intermediate between Penaeidae and Aristeidae, and (b) Burkenroad, 1983, reconstructed from key (mentioned by the author as "...a natural key down to the level of genus", **relationships of solenocerid genera after Pérez Farfante, 1977 who mostly based on the grouping of Burkenroad (1936); genera in brackets were recently discovered or split from existing genera). # Considered as the most primitive group. ## Considered as the most advanced group. "?" refers to uncertain relationship. Noted that all names used here follow Pérez Farfante and Kensley (1997), with many of them different from those used by Kubo (1949) and Burkenroad (1983), and they both did not recognize the five-family scheme in Penaeoidea. (c) Phylogeny of the dendrobranchiate families based on sperm ultrastructure proposed by Scelzo and Medina (2004) and Medina et al. (2006a, b).

relatively few characters assessed, these results should be viewed as preliminary in terms of phylogenetic reconstruction. On the other hand, molecular phylogenetic studies have produced controversial results that partly refute the monophyly and long-established classification of the penaeoid families. A study based on mitochondrial 16S rRNA gene sequences found Penaeidae paraphyletic, with Solenoceridae nested within it, whereas the other three families are closely related and not reciprocally monophyletic (Vázque-Bader et al., 2004). Subsequent phylogenetic analysis using both 16S and another mitochondrial gene cytochrome *c* oxidase subunit 1 (COI) consistently showed the insertion of Solenoceridae into Penaeidae (Quan et al., 2004; Voloch et al., 2005). Nonetheless, bootstrap supports for the inferred topologies are weak and the taxon sampling was limited in these studies. It therefore remains unclear whether the contrasting results represent discrepancies between character evolution and speciation or artifacts of gene tree reconstruction. A more comprehensive study using markers that confer better resolution is needed to decide between these alternative hypotheses on the evolution of Penaeoidea.

1.2.2 Intergeneric phylogeny of Penaeidae

Penaeidae, with about 200 species known to date, is the most species-rich penaeoid family. These penaeids populate every ocean on earth and have the highest diversity in the Indo-west Pacific. As a family of shrimps with high economical importance, the recent taxonomic revision by Pérez Farfante and Kensley (1997) that split the family into 26 genera from an old 17-genera scheme by Dall and colleagues (1990) has instigated much debate. Comprehensive study on the phylogenetic relationship

of these genera has been limited. Based on morphology of the penaeids, only two very different schemes have ever been proposed. Kubo (1949) separated the family (then as subfamily Penaeinae) into five groups (without proper naming) and only suggested that the lineage harboring *Penaeus* and *Miyadiella* as basal (Figure 1.2a). Burkenroad (1983) separated the family (as a subfamily Penaeinae) into three tribes, namely Penaeini, Parapenaeini and Trachypenaeini, and placed Penaeini at the basal position (fig 1.2b).

These two competing hypotheses remained untested until decades later when several phylogenetic studies using mitochondrial gene sequences (16S and/or COI) provided support for Burkenroad's three-tribe scheme (Vázquez-Bader et al., 2004; Quan et al., 2004; Voloch et al., 2005). Some of these studies, however, did not find Penaeini to be basal and the situation was further complicated by the close relationship between Solenoceridae and Parapenaeini inferred from these studies. Nonetheless, the branch supports in these phylogenetic trees are not convincing, possibly due to the limited sampling of genera of Penaeidae. Recently, a comprehensive phylogenetic study was carried out encompassing 20 of the 26 genera (Chan et al., 2008). The phylogenetic tree based on 16S sequences generally supports the three-tribe scheme proposed by Burkenroad (1983) and also provides evidence for a basal Penaeini. Yet there are still some obscurities for the full support of this scheme: two members of Trachypenaeini (*Atypopenaeus* and *Trachypenaeopsis*) grouped with Parapenaeini and Penaeini respectively, making Trachypenaeini polyphyletic.

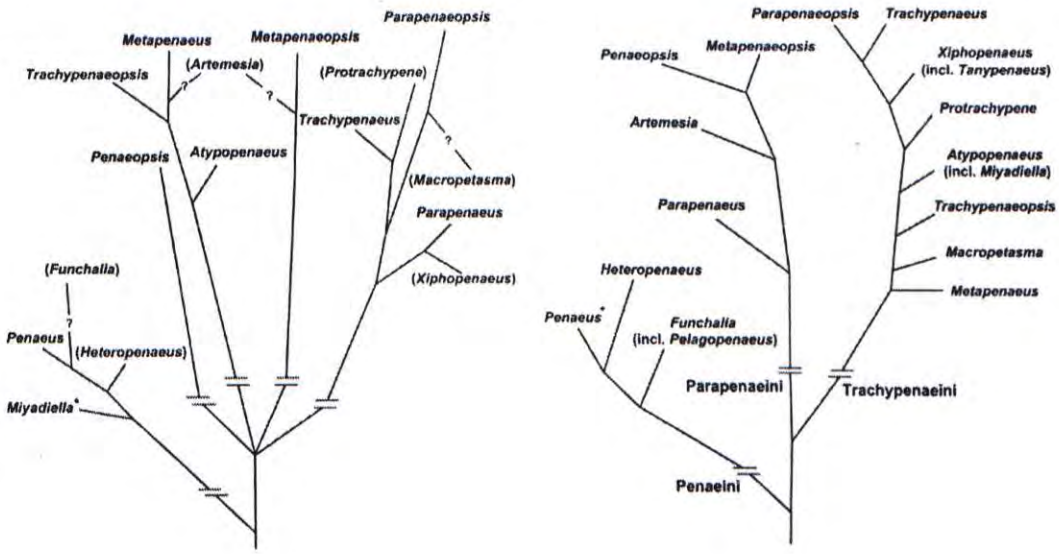

Figure 1.2. Morphological phylogeny of the penaeid genera proposed by (a) Kubo, 1949, reconstructed from text (genera in brackets not fully analyzed and ? referring to uncertain relationship) and (b) Burkenroad, 1983, reconstructed from key (mentioned by the author as "...a natural key down to the level of genus."), with Penaeini as Peneini, Parapenaeini as Parapeneini, Trachypenaeini as Trachypeneini, and *Metapenaeus* as *Mangalura*. *considered as the most primitive genus in the family. Adopted from Chan et al. (2008) with permission from the authors.

1.2.3 Interspecific phylogeny of Penaeus s.l.

Within the family Penaeidae, the *Penaeus* shrimps represent the most economically important fishery and aquaculture products among shrimps (or even crustaceans) worldwide (Chan, 1998; Dall et al., 1990; Holthuis, 1980; Pérez Farfante and Kensley, 1997; Rosenberry, 2001). To date, 28 *Penaeus* species are recognized and their phylogeny has attracted the most interest among all the penaeid shrimps. Studies on phylogenetic relationships among *Penaeus senus lato* species had been fueled by a controversial taxonomic revision by Pérez Farfante and Kensley (1997) in which the six subgenera of *Penaeus* shrimps were raised to generic level (fig. 1.3). The history of these subgenera goes back to Burkenroad (1934) who divided the genus into "grooved carapace" and "non-grooved carapace" lineages in which a grooved carapace was generally regarded as derived. Kubo (1949) divided the "non-grooved" lineage into two groups: with or without hepatic ridge. While the latter was given a subgeneric name of *Fenneropenaeus* by Pérez Farfante (1969), those with hepatic ridge were further separated as *Litopenaeus* (with open thelycum, usually regarded as ancestral) and *Penaeus* (with closed thelycum). From the "grooved carapace" lineage (subgenus *Melicertus*, Pérez Farfante 1969), Tirmizi (1971) isolated a single species as subgenus *Marsupenaeus*, which possesses peculiar tube-like thelycum. Burukovsky (1972) divided the "grooved" shrimps in America (and named as *Farfantepenaeus*) from *Melicertus*, which inhabit the Indo-West Pacific (except *M. kerathurus* which inhabits the east Atlantic). Although these subgenera had long been established in 1997 when Pérez Farfante and Kensley elevated them to generic rank, reception to this change has been mixed; some accepted it while others rejected it out of hand. The focuses of the controversy were primarily on

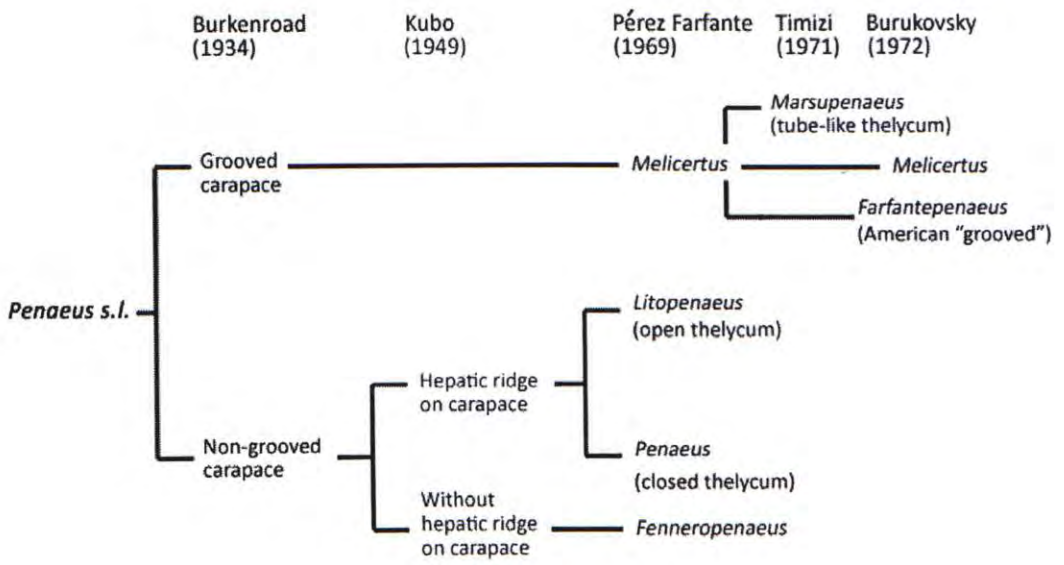

Figure 1.3. Morphological phylogeny of *Penaeus s.l.*

whether or not the new classification truly reflects the phylogenetic relationships of the *Penaeus* species and on what taxonomic rank should be assigned to the different lineages within *Penaeus s.l.* (Dall, 2007; Flegel, 2007, 2008; McLaughlin et al., 2008). Those who refused the change felt that there were insufficient morphological evidences to support monophyly in the proposed taxa (Davie, 2002; Flegel, 2008), and many of these skeptics have resorted to molecular approaches to resolve this dilemma.

Baldwin et al. (1998) studied the phylogenetic relationships of 13 *Penaeus* s.l. species using mitochondrial COI sequences and found evidences that challenged the monophyly of *Melicertus*, *Penaeus sensu stricto*, *Litopenaeus* and *Farfantepenaeus*. The lineage containing *Melicertus* and *Marsupenaeus* occupied the basal position in the COI gene tree. A similar result was obtained by Gusmao et al. (2000) based on both COI sequences (mostly adopted from Baldwin's study) and 11 isozyme loci. However, phylogenetic reconstruction using partial sequences of 16S rRNA (Maggioni et al., 2001) yielded contrasting results which strongly supported the monophyly of *Farfantepeaneus* and *Litopenaeus*. Nonetheless, a comprehensive view on relationships among species within each subgenus could not be well resolved from these studies owing to their constraints in taxon sampling and genetic characters. Therefore, Lavery et al. (2004) analysed concatenated 16S rRNA and COI sequences from 26 of 28 *Penaeus s.l.* species, and confirmed monophyly with high bootstrap support of all of the subgenera except for *Melicertus* (within which *Marsupenaeus* nested based on the COI + 16S dataset) and *Penaeus s.s.* (which appears to be paraphyletic with respect to *Fenneropenaeus*). Moreover, the division

of *Penaeus s.l.* into two clades (*Melicertus* + *Marsupenaeus* and *Fenneropenaeus* + *Farfantepenaeus* + *Litopenaeus* + *Penaeus s.s*) always received strong support. Therefore, if the taxonomic grouping is to reflect phylogenetic relationships, Lavery et al.'s data would support the division into these two natural clades but not the six subgenera as proposed by Pérez Farfante and Kensley (1997). The authors wisely suggest that further study, particularly using nuclear sequence data, is needed to ultimately confirm the systematics of these controversial taxa.

1.3 Molecular markers for phylogenetic studies of decapods

A fundamental concept in molecular phylogenetics is that life forms evolve by accumulating mutations in their genome, so that when we compare the divergence between DNA sequences from different organisms, we can estimate how recently they share a common ancestor (Brown, 2002) and hence, reconstruct their evolutionary history. A genome can contain over thousands of genes which can be employed as phylogenetic markers. However, with its own selection pressure, and hence mutation rate, each gene has a specific "optimal time frame" for phylogenetics inference, such that genes with lower mutation rate are more suitable for inferring more ancient relationships. In the subsequent part I will use molecular phylogenetics studies on decapods crustaceans to illustrate the applications of the commonly used molecular markers.

1.3.2 Mitochondrial markers

Since Cunningham et al. (1992) and Knowlton et al. (1993) employed gene sequences from the large ribosomal subunit 16S rRNA and the cytochrome *c* oxidase

subunit 1 (COI) in their pioneer mitochondrial DNA-based phylogenies on Crustacea, these two genes have dominated molecular phylogenetic studies of decapod crustaceans. Occasionally used in combination with these genes include the small ribosomal subunit 12S rRNA and the cytochrome *c* oxidase subunit 2 (COII).

Animal mitochondrial genome is 15-20 kb in length, composed of 37 genes coding for 22 tRNAs, 2 rRNAs and 13 mRNAs (Avise, 2004). Mitochondrial markers have been favored for phylogenetic studies for several reasons. Firstly, due to the fact that mitochondrial DNA is rendered more stable by its closed circular structure and because of the presence of thousands of mitochondria (and hence mitochondrial genomes) in a cell, it is much easier to extract large quantity of mitochondrial DNA and to amplify mitochondrial genes (Avise, 1998). Secondly, mitochondrial DNA is transmitted predominantly through maternal lines (Dawid and Blacker, 1972; Hutchison et al., 1974; Giles et al., 1980; Gyllensten et al., 1985; Avise and Vrijenhoek, 1987). This property limits the opportunity of genetic recombination among mitochondrial genomes, and therefore simplifies phylogenetic interpretation. Thirdly, mitochondrial DNA evolves rapidly, due in part to the presence of many free radicals and to the inefficient mutation repair mechanisms (Brown et al., 1979; Wilson et al., 1985). This allows phylogenetic signals to accumulate at a shorter time frame, rendering mitochondrial DNA sequences suitable for elucidating intrafamilial (e.g. Voloch et al., 2005; Chan et al., 2008) and intrageneric (e.g. Ptacek et al., 2001; Schubart et al., 2001; Braband et al, 2006; von Rintelen et al., 2007) relationships. Fourthly, universal primer sets for mitochondrial markers are available so that laboratory time for developing new primers can be much reduced. This convenience

has rendered mitochondrial markers to be used across a wide range of taxa of decapods, ranging from the dendrobranchiate shrimps (e.g. Lavery et al, 2004; Voloch et al., 2005) to lobsters (e.g. Ptacek et al., 2001; Braband et al., 2006), crabs (e.g. Schubart et al., 2001; Harrison, 2004) and hermit crabs (e.g. Mantellato et al., 2006).

Nonetheless, it is inappropriate to use mitochondrial DNA exclusively to elucidate ancient relationships, for instance, above family level (Schubart et al., 2000). This is because the high mutation rate of mitochondrial genes can result in substitution saturation and homoplasy, giving erroneous phylogenetic signals when older splits are analyzed. Exclusive use of mitochondrial gene sequences to reconstruct high taxonomic level relationships usually results in trees with low bootstrap supports and misleading topologies (e.g. Vázque-Bader et al., 2004).

1.3.3 Nuclear markers

Because of the limitation of mitochondrial DNA in scrutinizing ancient phylogenetics events, nuclear markers have been frequently employed, exclusively or in combination with mitochondrial markers, in studies of higher level phylogeny in decapods. Among nuclear markers, rRNA genes such as 18S and 28S are more commonly used (e.g. Pérez-Losada et al., 2004; Shull et al., 2005; Ahyong et al., 2007; Tsang et al., 2008a). Pioneering works by Kim and Abele (1990) and Spears et al (1992) have demonstrated the usefulness of nuclear rRNA genes in resolving phylogeny of decapods among infraorders and families. Their extensive applications were partly due to their relative ease to be amplified by PCR as there are hundreds of

copies of these genes in each genome. These genes are composed of a mixture of several hyperconserved and hypervariable regions (Simon et al., 1994). While the conserved regions may contain valuable information for resolving deep branches, the variable regions are often interspersed with indels of variable length, making it very difficult to align. This characteristic of nuclear rRNA genes has impeded their utility in phylogenetic studies because the reliability of sequence alignment is a critical factor in molecular phylogenetic analyses (Swofford et al., 1996).

Unlike rRNA genes, alignment of protein coding gene sequences can be confidently performed, thanks to their triplet codon arrangement. An additional advantage of nuclear protein coding genes is that they are informative across a wide range of taxonomic levels (Rokas et al., 2002). They have been commonly used in phylogenetic studies in insects (e.g. Wiegmann et al., 2000; Leyes et al., 2002; Danforth et al., 2004a,b). Their application to decapod phylogenetics, however, has been scarce until recently (histone H3 in Porter et al., 2005, and glyceraldehydes-3-phosphate dehydrogenase in Buhay et al., 2007), possibly due to our limited understanding on decapod genomes which has impeded the development of nuclear markers.

Recently, our laboratory has demonstrated the utility of two nuclear protein-coding genes, phosphoenolpyruvate carboxykinase (PEPCK) and sodium-potassium ATPase α-subunit (NaK), as molecular markers in elucidating decapod infra-ordinal phylogenetics (Tsang et al., 2008b). These two genes participate in fundamental cellular functions across the animal kingdom and are well-conserved throughout

evolution. Previously, they have been applied successfully to resolve deep-level phylogeny of insects (e.g. Friedlander et al., 1996; Leyes et al., 2002) and bilateral metazoans (Anderson et al., 2004). Given that these genes are informative across a wide range of taxonomic levels, I attempt to utilize PEPCK and NaK gene sequences as principal markers to investigate the phylogenetic relationships within Penaeoidea in this thesis research.

Chapter 2

Molecular phylogeny of superfamily Penaeoidea

2.1 Introduction

Shrimps in the superfamily Penaeoidea represent a group of marine fauna with high economic value. Attention to the phylogeny of Penaeoidea has been raised since the evolutionary relationships revealed by recent sperm ultrastructure (Scelzo and Medina, 2004; Medina et al., 2006a, b; see fig. 1.1c) and molecular approaches (Quan et al., 2004; Vázque-Bader et al., 2004; Voloch et al., 2005) contrast drastically with the traditional morphological studies, as mentioned in Chapter 1. These uncertainties in the relationships among the five penaeoid families have led to dispute in the long established taxonomy. As the resolutions in the previous sperm ultrastructure and molecular studies were lowered by their limited taxon sampling and inadequate morphological or molecular characters for analysis, a more comprehensive study using markers with better resolution is thus necessary to discern alternative hypotheses on the evolution relationships within Penaeoidea. Moreover, the phylogeny of genera in family Penaeidae also requires further investigation as preceding molecular studies have found obscurities in their relationships (See Chapter 1).

A thorough understanding of evolutionary history requires knowledge not only of phylogenetic relationships but also of the origin and diversification time of the taxa, which is essential for determining whether and how major geological or ecological

events impacted on the evolution of organisms. However, owing to their rare and incomplete fossil records, little is known about when penaeoid shrimps diversified. The first trace of penaeoids appeared in the Permo-Triassic period (Burkenroad, 1963; Glaessner, 1969), while the Triassic and Jurassic era were dominated by the family Penaeidae, which began to diversify in the Cretaceous (Glaessener, 1969; Garassino, 1994; Garassino and Teruzzi, 1994). Fossils of Sicyoniidae and Benthesicymidae have been discovered, although rarely, in Cretaceous deposits, but no relics of Aristeidae and Solenoceridae ancestors are recorded from the Mesozoic (Glaessner, 1969). Based on the observation that some recent solenocerids (e.g. *Haliporus*) possess several characters of the Jurassic fossil *Aeger* (Burkenroad, 1936; 1945; 1963), Burkenroad (1983) hypothesized that the ancestor of the dendrobranchiates may be more solenocerid-like, and that the solenocerid-like lineage should have a longer evolutionary history than Penaeidae.. However, the discovery of the more ancient Triassic fossil *Antrimpos* that closely resembles the extant *Penaeus* (Burkenroad, 1963; Glaessner, 1969) may suggest that the family Penaeidae had been established earlier than the solenocerid-like lineage. Unfortunately, the absence of fossil aristeids and solenocerids prohibits a direct delineation of these alternative hypotheses. Moreover, rapid diversification and radiation have commonly been observed in the fossil records of crustaceans (Schram et al., 1978), but the scarcity of Penaeoidea fossils makes it difficult to determine whether this phenomenon applies also to this group. Application of the relaxed molecular clock method, which permits variation of evolutionary rates across the tree and incorporation of fossil constraints in divergence time estimations, may shed more light on the problem of diversification (Drummond et al., 2006; Rutschmann,

2006). Nonetheless, a reliable phylogenetic tree is a prerequisite for accurate estimations.

In this part of the study, two nuclear protein-coding genes, phosphoenolpyruvate carboxykinase (PEPCK) and sodium-potassium ATPase α-subunit (NaK), were used to reconstruct the phylogeny of Penaeoidea as well as Penaeidae within it. These markers have been proven useful for decapods infra-ordinal phylogenetics (Tsang et al., 2008b). We aimed to test the alternative hypotheses on the familial relationships of the penaeoids which should, in anticipation, provide new insights to the evolution and classification of the group. We also estimated the divergence ages of the major taxa of Penaeoidea using the relatively more robust phylogenetic tree inferred from the nuclear protein-coding genes.

2.2 Materials and methods

2.2.1 Taxon sampling

We collected the penaeoid shrimps for this study either by trawling them directly from the sea or by purchasing them from fish markets. We followed the most recent classification scheme proposed by Pérez Farfante and Kensley (1997) throughout the study. Representatives from 36 of the 49 genera of the five families in Pérez-Farfante and Kensley (1997) were analyzed, including seven of nine genera in Aristeidae, two of four genera in Benthesicymidae, 18 of 26 genera in Penaeidae, the single genus of Sicyoniidae, and eight of nine genera in Solenoceridae, in a total of 45 species (table 2.1). Only one individual per species was analyzed. Specimen of *Trachypenaeopsis richtersii* which was used in our previous phylogenetic study

using mitochondrial genes (Chan et al., 2008) was also available for analyses, but PCR amplifications of the targeted genes in this sample were not successful. Three members of Sergestidae (*Acetes* sp., *Sergestes* sp. and *Sergia maxima*) which is the sister superfamily of Penaeoidea in the suborder Dendrobranchiata, together with a caridean *Rhynchocinetes durbanensis*, and an euphausidean *Euphausia superba*, were used as outgroup taxa. Species identification followed the keys of Crosnier (1978, 1988, and 2003), Liu and Zhong (1986), Yu and Chan (1986), Pérez Farfante (1988), Dall et al. (1990), Pérez Farfante and Kensley (1997) and Chan (1998). Identification of some aristeids, solenocerids and *Sicyonia* was verified by A. Crosnier. Samples were either frozen at -70°C or preserved at 95% ethanol prior to DNA extraction.

2.2.2. DNA extraction, PCR and sequencing

Total genomic DNA was extracted from pleopod muscle using the commercial QIAamp Tissue Kit (QIAGEN). Primers for amplification of PEPCK and NaK were based on Tsang et al (2008b). Amplifications were carried out in a reaction mix containing 1-5 μl of template DNA, 1X PCR reaction buffer, 3 mM $MgCl_2$, 200 μM dNTPs, 200 nM of each primer, 1.5 units of *Taq* polymerase (Amersham) and ddH_2O to a total volume of 50 μl. The PCR profile for both genes was as follows: 3 min at 94°C for initial denaturation, followed by 35 cycles of 30 s at 94°C, 30 s at 55-60°C (depending on individual samples), 1 min 30 s at 72°C with a final extension for 10 min at 72°C. The PCR products were purified using the QIAquick gel purification kit (QIAGEN) in accordance with the manufacturer's instructions. The same sets of primers were used in sequencing reactions conducted by an

Table 2.1. Classification, sampling locations and voucher ID of the species and GenBank accession number of the gene sequences of the present study.

Superfamily	Family	Species	Sampling location
Dendrobranchiata			
Penaeoidea	Aristeidae	*Aristaeomorpha foliacea*	Taiwan
		Aristaeopsis edwardsiana	Taiwan
		Aristeus mabahissae	Taiwan
		Aristeus pallidicauda	Taiwan
		Aristeus virilis	Taiwan
		Hemipenaeus carpenteri	Taiwan
		Hepomadus glacialis	Taiwan
		Parahepomadus vaubani	Taiwan
		Plesiopenaeus armatus	Taiwan
	Benthesicymidae	*Benthesicymus investigatoris*	Taiwan
		Benthonectes filipes	Is. Wallis
	Penaeidae	*Atypopenaeus dearmatus*	Philippines
		Farfantepenaeus aztecus	Gulf of Mexico
		Fenneropenaeus chinensis	Zhujiang estuary, China
		Fenneropenaeus merguiensis	Fish market, Hong Kong
		Funchalia sp.	Philippines
		Litopenaeus setiferus	Gulf of Mexico
		Litopenaeus vannamei	Fish market, Hong Kong
		Marsupenaeus japonicus	Singapore
		Megokris pescadoreensis	Taiwan
		Melicertus latisulcatus	Taiwan
		Metapenaeopsis palmensis	Fish market, Hong Kong
		Metapenaeopsis provocatoria longirostris	Taiwan
		Metapenaeus affinis	Fish market, Hong Kong
		Metapenaeus ensis	Fish market, Hong Kong
		Parapenaeopsis cornuta	Taiwan
		Parapenaeus sextuberculatus	Taiwan
		Pelagopenaeus balboae	Indian Ocean
		Penaeopsis eduardoi	Taiwan
		Penaeus monodon	Fish market, Hong Kong
		Rimapenaeus pacificus	Panama
		Trachysalambria starobogatovi	Natal, S. Africa
		Xiphopenaeus kroyeri	French Guiana
	Sicyoniidae	*Sicyonia lancifer*	Taiwan
		Sicyonia curvirostris	Taiwan
		Sicyonia fallax	Taiwan
	Solenoceridae	*Cryptopenaeus clevai*	Taiwan
		Gordonella paravillosa	Taiwan

Table 2.1 Continued

Superfamily	Family	Species	Sampling location
Penaeoidea	Solenoceridae	*Hadropenaeus lucasii*	Taiwan
		Haliporoides sibogae	Taiwan
		Haliporus taprobanensis	Taiwan
		Hymenopenaeus equalis	Taiwan
		Mesopenaeus brucei	Taiwan
		Solenocera melantho	Taiwan
		Solenocera crassicornis	Fish market, Hong Kong
Sergestoidea	Sergestidae	*Acetes* sp.	Fish market, Hong Kong
		Sergestes sp.	Philippines
		Sergia maxima	Taiwan
Caridea			
Nematocaricinoidea	Rhynchocinetidae	*Rhynchocinetes durbanensis*	Aquarium shop, Hong Kong
Euphausiacea			
Euphausidea	Euphausiidae	*Euphausia superba*	Fish market, Hong Kong

Applied Biosystems 3100 automated sequencer using the ABI Big-dye Ready-Reaction mix kit, following a standard cycle sequencing protocol.

2.2.3. Phylogenetic analyses

Nucleotide sequences were aligned using CLUSTAL W (Thompson et al., 1994) using default parameters, manually adjusted and confirmed by translating into amino acid sequences. The best-fit models of nucleotide substitution for both the concatenated dataset and individual genes were determined by Modeltest 3.7 (Posada and Crandall, 1998). The combined dataset was analysed under maximum likelihood (ML) using PhyML program (Guindon and Gascuel, 2003; Guindon et al., 2005; available at: http://atgc.lirmm.fr/phyml/). In ML analysis, two independent runs/analyses were performed with nodal support estimated from 1000 bootstrap (BP) pseudoreplicates. For the combined data set, the data was partitioned by gene and separate models were assigned to each partition in the Bayesian inference (BI) analysis implemented in MrBayes v.3.12 (Ronquist and Huelsenbeck, 2003). Datasets of each gene were also analysed using the BI. Three independent runs were carried out with four differentially heated Metropolis coupled Monte Carlo Markov Chains for 5,000,000 generations started from a random tree. Model parameters were estimated during both ML and BI analyses. Chains were sampled every 500 generations and the trees before convergence were discarded as burn-in to ensure that analysis had stabilized (determined using Tracer v1.4, Rambaut and Drummond, 2004). Convergence was confirmed by monitoring likelihood values graphically. A 50% majority-rule consensus tree was constructed from the remaining trees to estimate posterior probabilities (PP).

Alternative phylogenetic hypotheses from previous morphological and molecular studies were tested using the Kishino–Hasegawa (KH) test (Kishino and Hasegawa, 1989) and Shimodaira–Hasegawa (SH) test (Shimodaira and Hasegawa, 1999) implemented in PAUP*. Alternative tree topologies were constructed using MacClade 3.0 (Maddison and Maddison, 1992) by rearranging the branches showing conflicting relationships between the ML tree and the a priori hypotheses. The tests were carried out with RELL optimization and 1000 bootstrap pseudoreplicates.

2.2.4. Divergence time estimation

BEAST v1.4.7 (Drummond and Rambaut, 2007) was used to estimate the divergence times of all nodes. This Bayesian method employs a relaxed molecular clock model, allowing evolutionary rates to vary in different lineages, and permits multiple constraints deduced from fossil records.

Fossil records of the penaeoids were limited. Only four fossil constraints could be placed on the analysis of divergence times. (1) The earliest dendrobranchiate fossils were found in the Triassic deposits in Europe and Madagascar and these included two families of Penaeoidea (Glaessner, 1969; Burkenroad, 1963, 1981). The *Antrimpos* fossils are "quite indistinguishable from the living *Penaeus*" (Burkenroad, 1981) but many fossil species not showing diagnostic characters of recent Penaeidae have tended to be assigned to this genus (Balss, 1922). For a cautious estimation, we regard it as an ancestral stock of Penaeidae. The *Aeger* fossils constitute an extinct family, Aegeridae (Burkenroad, 1963), that once existed from the Triassic to the late

Cretaceous era (Glaessner, 1969; Feldmann et al., 2007). The existence of two distinct families of Penaeoidea in the Triassic implies that the superfamily had diverged prior to that period. Therefore, a log normal (zero offset = 248 MYA, SD = 1) prior distribution was implemented to place the most recent common ancestor (MRCA) of Penaeoidea at the end of the Permian era. (2) The most ancient fossils of the *Penaeus sensu lato* (i.e., containing *Farfantepenaeus*, *Fenneropenaeus*, *Litopenaeus*, *Penaeus sensu stricto*, *Marsupenaeus* and *Melicertus*; Pérez Farfante and Kensley, 1997) were discovered in the Jurassic shale (Glaessner, 1969; Dall et al., 1990). They are more commonly found in the Cretaceous and had a record from India in the lower Tertiary (Dall et al., 1990). Hence a constraint on the divergence of *Penaeus* s.l. was placed before the end of the Jurassic period (log normal prior, zero offset = 144 MYA, SD = 1). (3 and 4) The oldest fossils of *Sicyonia* and *Bentheysicymus* were discovered in Cretaceous shales (Glaessner, 1969). Constrains of 65 MYA with SD =1 were set on the divergence of both *Sicyonia* and Benthesicymidae respectively. The log normal prior distribution was chosen for all fossil constraints because it assumes that the divergence time should predate the fossil occurrence, and that the probability of divergence should be highest on the fossil age and decrease towards earlier period (Leaché and Mulcahy, 2007).

The models for the gene-partitioned datasets were chosen by Modeltest 3.7. The uncorrelated lognormal relaxed molecular clock model with a Yule prior distribution for branching rates was employed. All of the Markov chain Monte Carlo analyses were run for 10 million generations with a burnin of one million generations and sampled every 1000 generations. The analyses were repeated to refine the tuning

operators to improve efficiency using the auto-optimize function in BEAST. Two separate runs were then combined and Tracer v1.4 was used to determine the effective sample size of each parameter (Rambaut & Drummond 2004).

2.3 Results

2.3.1 Phylogenetic analyses

The aligned partial sequences of PEPCK gene included 570 nucleotide positions with 217 parsimony informative sites. The NaK gene included 582 positions in which 209 were parsimony informative (table 2.2). No introns or indels were observed. Ambiguous sites (double peaks in chromatograms), probably due to heterozygosity of individuals, were coded as ambiguous using the IUB symbols. Sequences of PEPCK were slightly GC rich (56.4%) while those of NaK showed small AT bias (51.3%). However there was no significant base heterogeneity across all codon positions of the two genes (Chi-square $p = 0.4878$) (table 2.2). The Kimura 2-parameter distance matrixes of PEPCK and NaK sequence data are shown in tables 2.3 and 2.4. Average interfamily distances of PEPCK and NaK ranged from 0.034 to 0.161 and 0.089 to 0.168 respectively. The pairwise distances within and among Aristeidae, Benthesicymidae and Solenoceridae appeared higher in NaK than in PEPCK while the opposite occurred in Sicyoniidae and Penaeidae. The interfamilial genetic distance was lowest between Aristeidae and Bentheysicymidae (only 0.034 in PEPK and 0.089 in NaK), while distances between tribes of Penaeidae (0.101-0.127 in PEPCK and 0.106-0.118 in NaK) were comparable or even higher than those among Aristeidae, Benthesicymidae and Solenoceridae (0.034-0.061 in PEPCK and 0.089-0.127 in NaK).

Table 2.2. Summary of parsimony results.

Gene	No. of sites	No. of variable sites	No. of parsimony informative sites	% A/T	Chi-square test (p)
PEPCK					
nt1	190	55	31	46.7	1
nt2	190	33	16	51.5	1
nt3	190	175	153	32.4	< 0.001
All sites	570	265	217	43.6	0.751
NaK					
nt1	194	56	37	44.5	1
nt2	194	33	12	61.6	1
nt3	194	176	160	47.9	< 0.001
All sites	582	265	209	51.3	1
Overall:					
Nucleotide	1152	528	409	47.5	0.4878

The Akaike Information Criterion implemented in ModelTest selected GTR + I + R as the best-fit model for the combined dataset in ML (base frequencies = 0.2521, 0.2872, 0.2351; Rmat = 1.5903, 4.0320, 1.7881, 1.4267, 6.0007; γ-shape parameter = 0.9364; proportion of invariable sites = 0.4352). The best-fit model for PEPCK dataset was HKY + I + G (base frequencies = 0.2551, 0.3318, 0.2157; T ratio = 1.4323; γ-shape parameter = 0.9746; proportion of invariable sites = 0.4579) while a SYM + I + G (Rmat = 1.7418, 4.4796, 2.3181, 1.1723, 8.5986; γ-shape parameter = 0.7770; proportion of invariable sites = 0.3961) was selected for NaK dataset.

The BI tree resulting from NaK (fig. 2.1) and PEPCK (fig. 2.2) sequences differ drastically. In the NaK gene tree, the relationships among family Aristeidae, Solenoceridae and Penaeidae were poorly resolved with low statistical support, but the gene tend to provide higher resolutions to the phylogeny of penaeid genera and strongly supported the incursion of Sicyoniidae into Penaeidae. The PEPCK gene tree, in contrast, offer low resolution in almost all relationships and only the grouping of some closely related genera/species received high support. Nonetheless when using the concatenated data set, the tree topologies resulting from ML and BI approaches were largely congruent and received high supports in most nodes. Only the relationships of several genera were poorly resolved and received low supports for their grouping. Here only the BI tree was presented (fig. 2.3) with support values for both BI and ML analyses. The most significant difference between the two tree topologies was that in the ML tree *Funchalia* sp. and *Pelagopenaeus balboae* were distantly related to *Penaeus s.l.* (not shown), while in the BI tree these two species

Table 2.3. Ranges of K2P distances of PEPCK gene within (italic, on diagonal) and between (below diagonal) families (and tribes of Penaeidae) of Penaeoidea with the average values in parentheses.

Family/tribe	Aristeidae	Benthesicymidae	Solenoceridae	Sicyoniidae	Penaeidae	Trachypenaeini (Penaeidae)	Penaeini (Penaeidae)	Parapenaeini (Penaeidae)
Aristeidae	*0.002 - 0.036 (0.021)*							
Benthesicymidae	0.031 - 0.038 (0.034)	*0.025 (0.025)*						
Solenoceridae	0.038 - 0.094 (0.061)	0.046 - 0.082 (0.061)	*0.017 - 0.074 (0.044)*					
Sicyoniidae	0.127 - 0.186 (0.161)	0.144 - 0.169 (0.156)	0.137 - 0.178 (0.155)	*0.007 - 0.152 (0.099)*				
Penaeidae	0.086 - 0.135 (0.11)	0.09 - 0.127 (0.111)	0.076 - 0.131 (0.104)	0.108 - 0.175 (0.147)	*0.015 - 0.151 (0.098)*			
Trachypenaeini (Penaeidae)	0.096 - 0.135 (0.117)	0.096 - 0.127 (0.112)	0.079 - 0.131 (0.105)	0.108 - 0.173 (0.142)	-	*0.034 - 0.116 (0.078)*		
Penaeini (Penaeidae)	0.086 - 0.126 (0.107)	0.097 - 0.126 (0.114)	0.086 - 0.13 (0.11)	0.137 - 0.164 (0.151)	-	0.103 - 0.151 (0.127)	*0.015 - 0.084 (0.05)*	
Parapenaeini (Penaeidae)	0.087 - 0.116 (0.102)	0.09 - 0.11 (0.102)	0.076 - 0.104 (0.09)	0.133 - 0.175 (0.149)	-	0.08 - 0.13 (0.101)	0.082 - 0.129 (0.11)	*0.025 - 0.078 (0.059)*

Table 2.4. Ranges of K2P distances of NaK gene within (italic, on diagonal) and between (below diagonal) families (and tribes of Penaeidae) of Penaeoidea with the average values in parentheses.

Family/tribe	Aristeidae	Benthesicymidae	Solenoceridae	Sicyoniidae	Penaeidae	Trachypenaeini (Penaeidae)	Penaeini (Penaeidae)	Parapenaeini (Penaeidae)
Aristeidae	*0.01 - 0.099 (0.053)*							
Benthesicymidae	0.069 - 0.112 (0.089)	*0.061 (0.061)*						
Solenoceridae	0.098 - 0.138 (0.119)	0.106 - 0.141 (0.127)	*0.023 - 0.102 (0.076)*					
Sicyoniidae	0.153 - 0.186 (0.166)	0.16 - 0.18 (0.168)	0.132 - 0.196 (0.158)	*0 - 0.047 (0.031)*				
Penaeidae	0.123 - 0.179 (0.144)	0.117 - 0.165 (0.141)	0.1 - 0.198 (0.134)	0.084 - 0.158 (0.124)	*0.008 - 0.153 (0.095)*			
Trachypenaeini (Penaeidae)	0.132 - 0.179 (0.149)	0.139 - 0.165 (0.153)	0.123 - 0.198 (0.148)	0.084 - 0.129 (0.111)	-	*0.026 - 0.116 (0.082)*		
Penaeini (Penaeidae)	0.123 - 0.157 (0.14)	0.117 - 0.142 (0.131)	0.1 - 0.166 (0.123)	0.122 - 0.158 (0.137)	-	0.096 - 0.153 (0.118)	*0.008 - 0.076 (0.045)*	
Parapenaeini (Penaeidae)	0.126 - 0.165 (0.144)	0.13 - 0.154 (0.142)	0.114 - 0.172 (0.134)	0.105 - 0.131 (0.116)	-	0.086 - 0.135 (0.109)	0.088 - 0.124 (0.106)	*0.046 - 0.083 (0.069)*

nested with a clade composed of *Marsupenaeus japonicus* and *Melicertus latisulcatus*, making *Penaeus s.l.* paraphyletic. However the supports at this position were low in both phylogenetic approaches and further study incorporating more markers and taxa of tribe Penaeini will be described in Chapter 3. Monophyly was evidenced with strong nodal support for the superfamily Penaeoidea and four of its families, Aristeidae, Benthesicymidae, Sicyoniidae and Solenoceridae. However, Penaeidae was paraphyletic with Sicyoniidae nested within it and the a priori hypothesis of Penaeidae monophyly was rejected by both KH and SH tests ($P < 0.05$). Our results did not support the close relationship among Aristeidae, Benthesicymidae and Sicyoniidae (KH and SH $P < 0.001$) that was suggested by mitochondrial markers, nor did they agree with the affinity of Solenoceridae to Penaeidae (without the incursion of Sicyoniidae, as proposed according to mtDNA and sperm morphology) (KH and SH $P < 0.001$). The five families were grouped into two clades, with clade A consisting of Solenoceridae, Aristeidae and Benthesicymidae (the latter two being sister taxa), and clade B including Penaeidae and Sicyoniidae.

2.3.1.1 Solenoceridae, Aristeidae *and* Benthesicymidae

It was strongly supported that Solenoceridae was distantly related to the other two families in clade A, but the relationships among the solenocerid genera were not well resolved. The family was divided into two clades, in which *Haliporides*, *Haliporus* and *Cryptopenaeus* appeared to be closely related. In the other clade, that *Gordonella* may be the most distantly related while *Hymenopenaeus* and *Hadropenaeus* were sister genera although the ML support was only moderate.

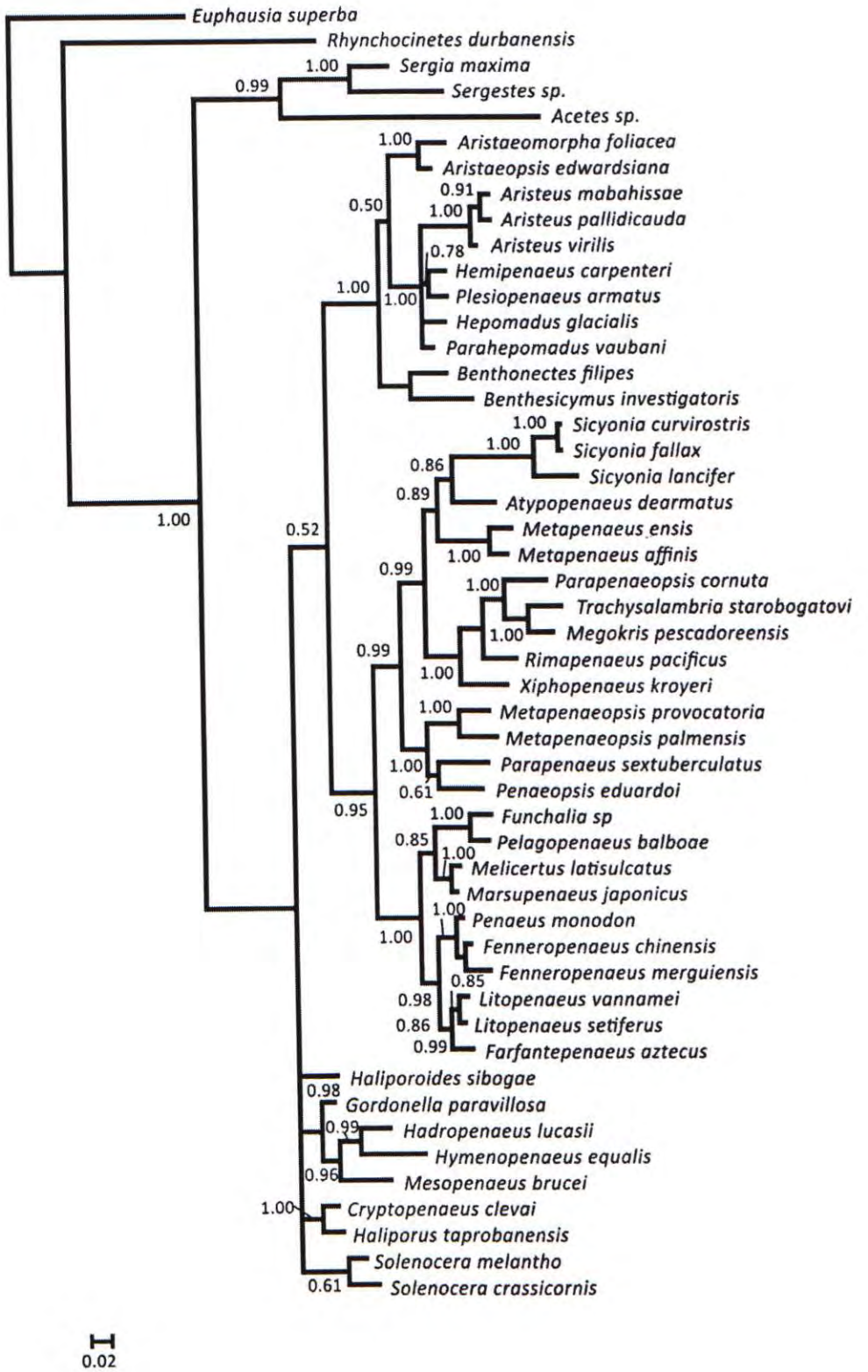

Figure 2.1 Bayesian inference tree from NaK analysis under the best-fitting model SYM + I + G. Numbers indicate posterior probabilities. Values below 50 are not shown.

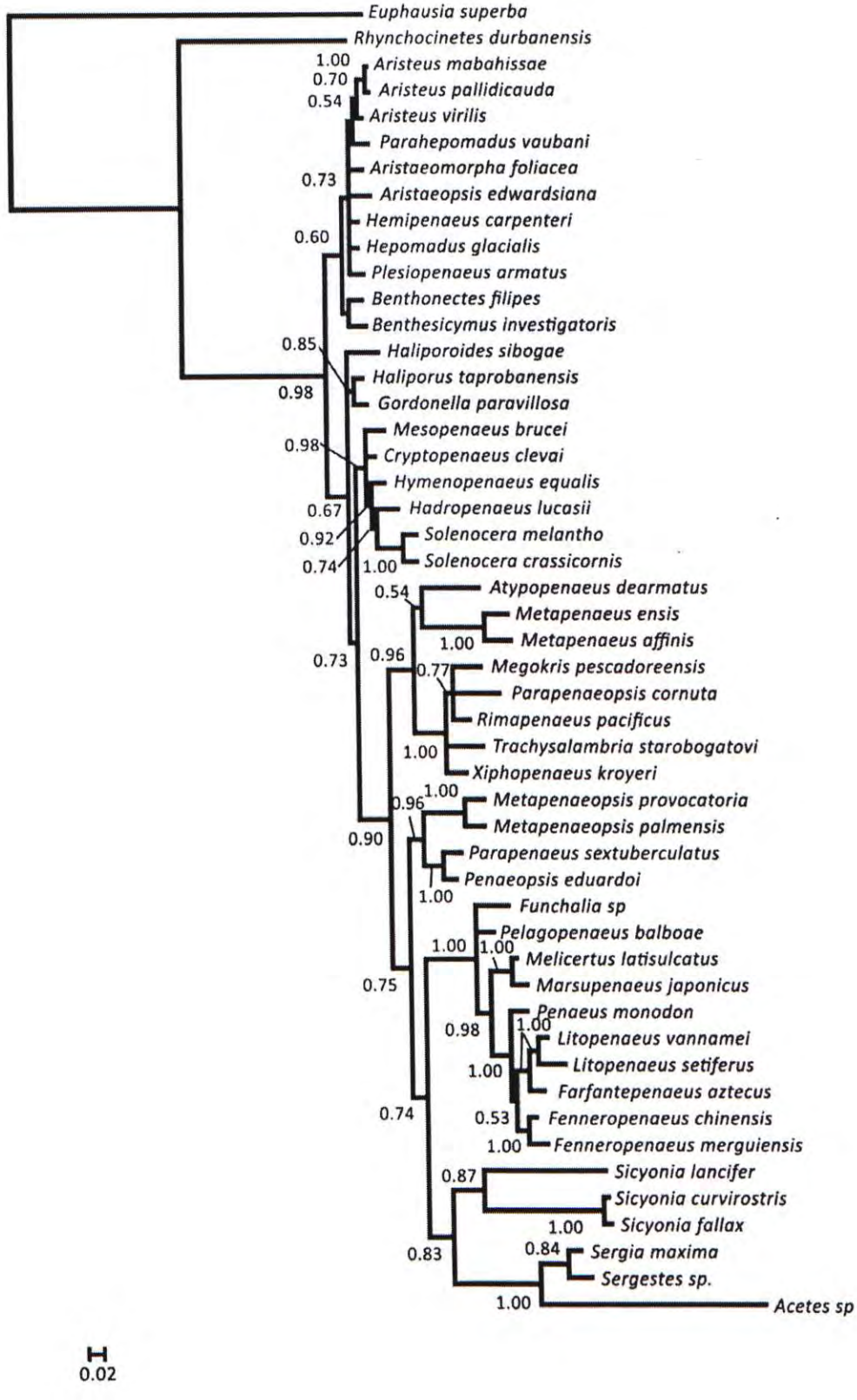

Figure 2.2 Bayesian inference tree from PEPCK analysis under the best-fitting model HKY + I + G. Numbers indicate posterior probabilities. Values below 50 are not shown.

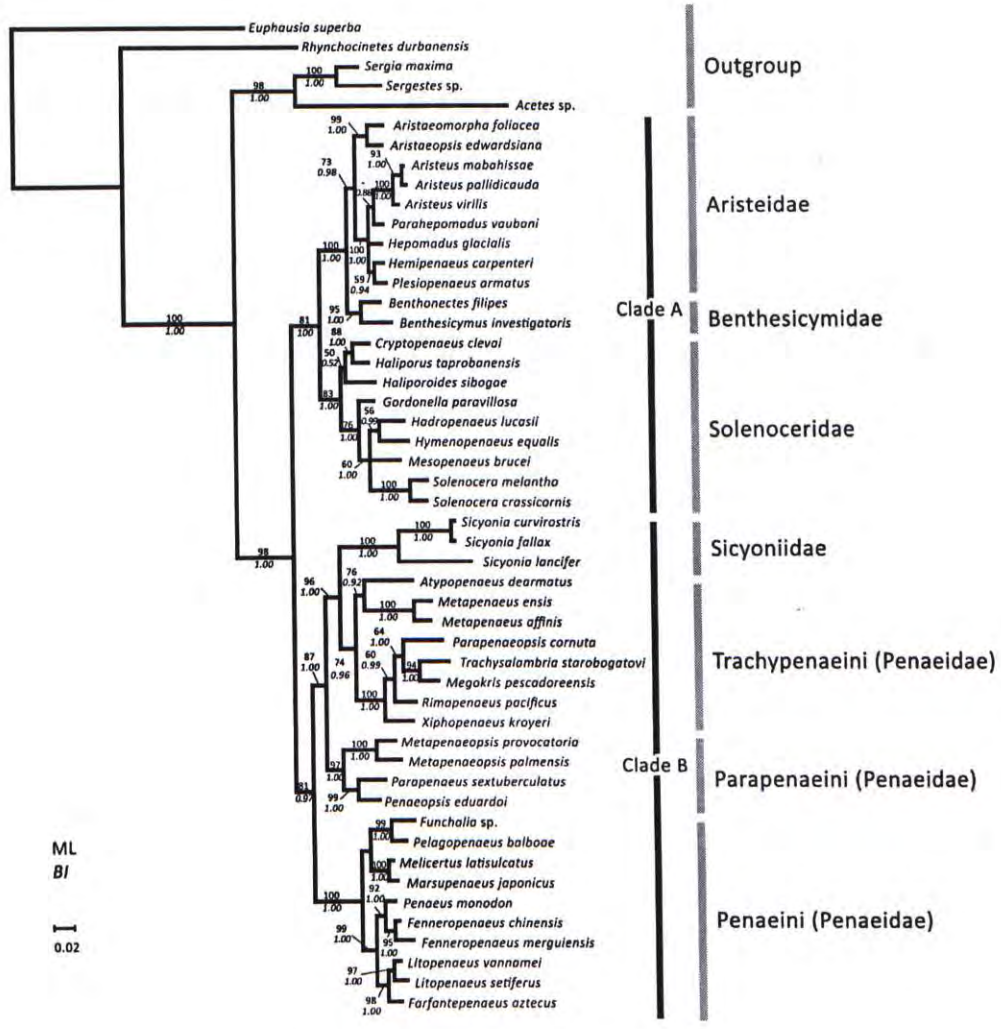

Figure 2.3. Bayesian inference tree from combined PEPCK and NaK analysis under the best-fitting model GTR+I+G. Numbers above branches indicate bootstrap values from maximum likelihood while posterior probabilities from BI are indicated below branches. Values below 50 are not shown.

Nonetheless, the position of *Mesopenaeus* was unclear and required further investigation. Benthesicymidae and Aristeidae were closely related as indicated by their low interfamily divergence (lowest among among all the major clades), but they are reciprocally monophyletic. Within Aristeidae, *Aristaeomorpha* and *Aristaeopsis* were closely related. The phylogeny of the remaining genera was obscure. *Parahepomadus* appeared to be sister to *Aristeus* while *Hemipenaeus* seemed to be sister to *Hymenopenaeus*. Nonetheless, ML bootstrap supports on these relationships were low despite the high PP from BI.

2.3.1.2 Penaeidae and Sicyoniidae

Three lineages of the penaeid genera were recovered with strong support, and were equivalent to the three tribes nominated by Burkenroad (1983), namely, Penaeini (as Penaeini including *Funchalia*, *Pelagopenaeus*, *Heteropenaeus* and *Penaeus sensu lato*), Parapenaeini (as Parapeneini including *Parapenaeus*, *Penaeopsis* and *Metapenaeopsis*) and Trachypenaeini (as Trachypeini including the remaining genera of the familiy). The molecular tree found tribe Penaeini most distantly related to the rest of the family while tribe Trachypenaeini was sister to Sicyoniidae. Tribe Parapenaeini was intermediate. These four lineages in clade B were genetically highly differentiated (table 2.3), with divergence levels comparable to those among the families in clade A. While the phylogenetic relationships among Penaeini genera cannot be clearly determined in this part of the study and will be further discussed in Chapter 3, phylogeny of Parapenaeini was well resolved with Parapenaeus appeared closer to Penaeopsis. In Trachypenaeini, *Atypopenaeus* and *Metapenaeus* grouped

together and formed a clade basal to the rest of the tribe. In the larger Trachypenaeini clade, *Xiphopenaeus* was the most distantly related, followed by *Rimapenaeus*. *Trachysalambria* and *Megokris* represented the most derived groups.

2.3.2 Divergence time estimation

In each MCMC run of 10 million generations in BEAST v1.4.7, the effective sample sizes of all parameters were well higher than optimal (data not shown) ensuring that the chains of the analyses were run long enough. Divergence dates estimated are shown in fig. 2.4 with 95% credibility intervals and posterior mean ages. The analyses showed that clade B diversified earlier at about 224 MYA while clade A split to give Aristeidae and Solenoceridae at about 174 MYA. The three tribes of Penaeidae were old, each with their tMRCA estimated to be over 126 MYA. They were older than the families Aristeidae (~79 MYA), Benthesicymidae (~69 MYA) and Solenoceridae (~120 MYA). Sicyoniidae was the youngest (~71 MY) of the four lineages in clade B, while tribe Penaeini radiated the earliest (~147 MY). Diversification of all of the aristeid and solenocerid genera appeared to occur within a shorter time frame (28-40 MYA and 48-93 MYA respectively when compared with those in clade B. Lineages in clade B radiated progressively over a period of more than a hundred million years. Although beyond the scope of this study, Dendrobranchiata is estimated to have diverged from the rest of Decapoda at about 473 MYA.

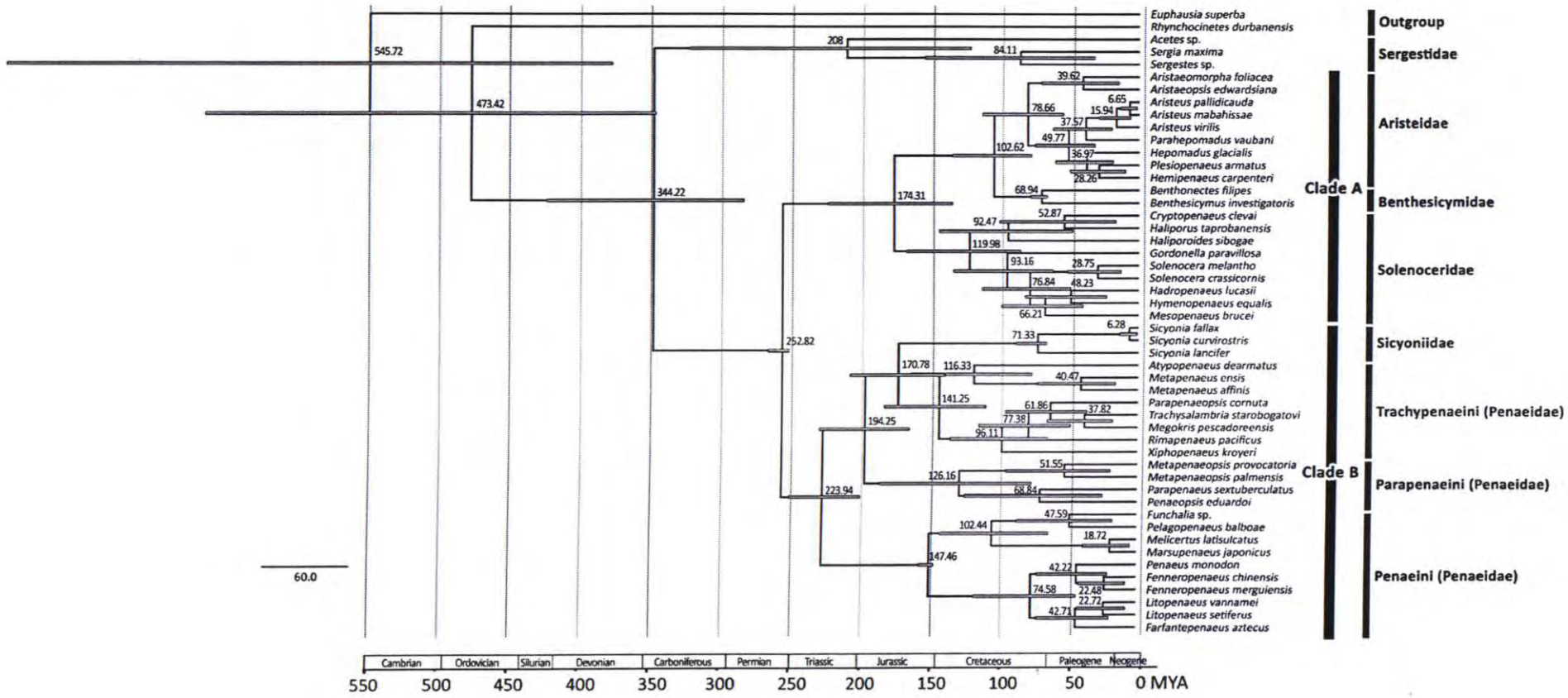

Figure 2.4. Phylogenetic tree showing molecular divergence estimates in millions of years based on a relaxed phylogenetic analysis of concatenated sequence data with grey bars showing 95% credibility intervals and posterior mean age adjacent to each node.

2.4 Discussion

2.4.1 Evolutionary relationships of the penaeoid shrimps

Our study presents the most comprehensive and robust molecular phylogenetic study of Penaeoidea to date. It is also the first molecular phylogenetic study to incorporate an extensive number of genera from Aristeidae and Solenoceridae, and thus can provide new fundamental information on the evolution of these families. The resulting phylogenetic tree is very different from those obtained from mitochondrial markers, which suggest a close relationship between Aristeidae, Benthesicymidae and Sicyoniidae (Vázque-Bader, 2004), in addition to the incursion of Solenoceridae within Penaeidae (Quan et al., 2004; Vázque-Bader et al., 2004; Voloch et al., 2005). However, the data supplied by mitochondrial genes, although suitable for the phylogenetics of closely related taxa, must be used with caution in resolving deep nodes because it is subject to a high level of homoplasy resulting from extreme compositional biases, asymmetry of transformation-rate matrices and rapid substitution saturation (Springer et al., 2001; Lin and Danforth, 2004). By contrast, nuclear protein-coding genes, such as PEPCK and NaK used in this study, were demonstrated to be informative across taxonomic levels (Rokas et al., 2002) and can provide good resolution to Mesozoic to Paleozoic-age systematics (Friedlander et al., 1996). Moreover, a more extensive taxon sampling in this study (when compared to only two genera from each family other than Penaeidae in previous molecular studies) gives better phylogenetic accuracy (Pollock et al., 2002; Zwickl and Hillis, 2002) and hence the tree stability and statistical support, than previous studies using mitochondrial markers, particularly at deeper branches. Our results, though fairly similar to Burkenroad's (1983) morphology-inferred phylogeny of the penaeoids,

propose yet another classification scheme for these shrimps.

The close associations of Penaeidae with Sicyoniidae (clade B), and Aristeidae with Benthesicymidae and Solenoceridae (clade A), have long been recognized in traditional taxonomy (e.g. Burkenroad, 1934, 1936, 1983; Crosnier, 1978). Crosnier (1978) and Burkenroad (1983) even believed that Penaeoidea had only two families, namely Aristeidae and Penaeidae. Although the association of Solenoceridae with Aristeidae (including Benthesicymidae) has been commonly accepted, phylogenetic studies based on sperm morphology and mitochondrial markers have found that Solenoceridae is closely related to Penaeidae. However, the limited taxon sampling of solenocerids might have caused erroneous results in these studies. Moreover, the phylogenetic inference based on mitochondrial DNA (Quan et al., 2004; Vázque-Bader et al., 2004; Voloch et al., 2005) might also be flawed due to mutational saturation as a result of the high mutation rates of these genes. On the other hand, the gain of spiked acrosome in sperms might have occurred several times independently throughout the evolution of dendrobranchiates, and hence might not necessarily be a synapomorphic character uniting Solenoceridae and Penaeidae.

The four lineages recovered from clade B are traditional in some respects and novel in others. Our results support the traditional three-tribe scheme of Burkenroad (1983): Penaeini, Trachypenaeini and Parapenaeini, with Penaeini as the basal tribe. A previous study using mitochondrial 16S rRNA gene sequences (Chan et al., 2008) also supports the three-tribe scheme. Yet the 16S gene tree places two Trachypenaeini genera, *Atypopenaeus* and *Trachypenaeopsis* into tribes

Parapenaeini and Penaeini respectively with weak support. The present study clearly shows that *Atypopenaeus* belongs to the Trachypenaeini, but the position of *Trachypenaeopsis* remains questionable as we did not obtain sequences from this genus. It is surprising, however, to find Sicyoniidae to be the sister taxon of this tribe, and as such nested within Penaeidae. Sicyoniidae is unique in Penaeoidea in that the posterior three pleopods are uniramous (vs. normal biramous pleopods in other penaeoids, as well as in carideans and lobsters) and it also has some other distinctive characters (see Burkenroad, 1983; Pérez Farfante and Kensley, 1997; Crosnier, 2003). However, the shape of the genitalia of Sicyoniidae, particularly the very rigid and strongly ridged petasma of males, is quite similar to many genera of Trachypenaeini. Burkenroad (1983) argued that Sicyoniidae have genitalia resembling those of Penaeini, but the petasma of the latter are lamella-like and rather thin.

2.4.2 Divergence dating and evolution of Penaeoidea

This study presents the first molecular dating of divergence events within Penaeoidea, and thus suggests new hypotheses on how paleo-geography, climate and ecology might have shaped the evolution of the superfamily. Fossil record suggests that the two lineages of Penaeoidea (clades A and B) might have diverged in the late Permian (253 MYA). These lineages have different preferences in adult habitats: the aristeid-like lineage (clade A) mostly inhabits deep ocean floor whereas the penaeid-like lineage (clade B) prefers shallow continental shelves (Burkenroad, 1934, 1936; Pérez Farfante, 1977; Dall et al., 1990). As its sister superfamily Sergestoidea also includes both deep-water and epipelagic shrimps, it is difficult to determine

conclusively whether the ancestors of penaeoids lived in littoral or bathyal zone. However, since fossil records indicate that Palaeozoic crustaceans predominantly inhabited shallow marine environment in the tropical Laurentia region (Schram, 1977), it is likely that penaeoids also have a shallow-water origin in Laurentia, from which the aristeid-like lineage evolved progressively to offshore environment. A similar "onshore-innovation, offshore-archaic" evolutionary shift has been postulated for various marine organisms such as the Cambrian-Ordovician marine benthic communities, late Cretaceous shelf fauna and the echinoderms (Jablonski et al., 1983; Jablonski and Bottjer, 1990). Populations inhabiting different depths might have experienced local selection pressures that isolate gene pools, such as differential effect of hydrostatic pressure on enzyme structure and function (Hochachka and Somero, 1984; Somero, 1990) and protein conformation, especially for those present on sperm and egg surfaces that influence reproductive compatibility (Chase et al., 1998). These local selection pressures might have led to ecological speciation in the ancestral stock of Penaeoidea in the Permian, resulting in lineages with dissimilar bathymetric affinity. Another plausible scenario is that the ancestral stock was sundered geographically, possibly due to the suturing of Pangea in the mid Permian and low sea level during most of the Permian and Triassic periods (Schram, 1977; Miller et al., 2005), resulting in allopatric speciation. In this case, the development of bathymetric adaptation might have occurred during population isolation or after re-mixing of the populations as the sea level rose in the Jurassic period. In either case the acquisition of new adaptation should have played an important role in the evolution of the penaeoid lineages.

The two existing phylogenetic schemes for penaeoids disagree on the origin of the group (fig. 1.1). Kubo (1949) regarded Sicyoniidae as the most primitive and Penaeidae as the most advanced, with Solenoceridae being somewhat falling midway between Penaeidae and Aristeidae. Burkenroad (1983), on the other hand, suggested that Solenoceridae (more precisely *Haliporus*) the most primitive and *Sicyonia* (and hence Sicyoniidae) is the most derived in Penaeoidea. Besides refuting the family grouping proposed by Kubo (1949), our results indicate that sicyoniids represent the most recent clade (excluding benthesicymids). Although these results support Burkenroad's view (1983) in regarding Sicyoniidae as the most advanced, they refute his hypothesis that solenocerids and aristeids diverged from each other earlier than penaeids and sicyoniids. Our study establishes that the penaeid-like lineage (clade B) started to radiate in the middle Triassic, preceding the aristeid-like lineage (clade A) which diverged in the middle Jurassic. The radiations of the five penaeoid families and the three tribes of Penaeoidea seem not to have been rapid. The time when the penaeid-like lineage began to diversify corresponds to the recovery period after the Permo-Triassic mass extinction, during which almost the entire Paleozoic fossil malacostracan fauna disappeared and might hence have created empty habitats for the radiation of the more advanced marine decapods that have dominated the oceans to the present day (Schram, 1977; Lopez-Gomez and Taylor, 2005). It has been proposed that unfavorable climatic and oceanographic conditions such as widespread anoxia and accumulation of greenhouse gases sustained for a long period after mass extinction, resulting in a lengthy recovery period when compared to other extinction events in the earth's history (Hallam, 1991; Kidder and Worsley, 2004). This may explain why the major groups in clade

B radiated in a progressive manner. On the other hand, the divergence of Solenoceridae from the Aristeidae-Benthesicymidae lineage in the middle Jurassic coincides with the splitting of Pangea.

The estimated divergence times of Penaeoidea and its families are comparable to those of other decapod taxa computed using similar methods. Superfamilies of other decapod infraorders are estimated to have radiated in the Permian (Porter et al., 2005), as Penaeoidea has been so estimated in this study. Porter et al. (2005) also noticed that the diversification of the astacid families occurred in the Cretaceous, and therefore shared the same time frame as the radiation of the penaeoid families Aristeidae, Benthesicymidae, Solenoceridae and Sicyoniidae. In addition, there is no significant difference between estimations of the age of divergence of Dendrobranchiata from Pleocyemata obtained by Porter et al. (2005) and the present study. We date the divergence back to the Ordovician period (473 MYA), slightly earlier than the Silurian radiation (437 MYA) estimated by Porter et al. (2005). The disparity may be due to the difference in fossil calibrations used or because only one dendrobranchiate species was analyzed in Porter et al.'s study so that the divergence between Dendrobranchiata and Pleocyemata might have been underestimated.

Although our results deduced from divergence age estimations are mostly in agreement with fossil records and the other molecular studies, they must be treated with a degree of caution due to several limitations. For instance, we have not taken into account some inherent inaccuracies associated with fossil ages such as misidentifications of the taxonomy of the fossils and inaccuracies in assigning the

fossils to geological strata (Graur and Martin, 2004). Moreover, errors might have crept into our calculations because we incorporated only a relatively small number of calibration points and used a single estimation method. Nonetheless we do not believe that limitations of this kind would significantly affect the thrust of our argument, and are confident that our main findings, viz. that the penaeid-like lineage was established earlier than the aristeid-like lineage, and that Penaeoidea did not undergo rapid radiation, are unlikely to be challenged.

2.4.3 Taxonomic revision

Given the paraphyly of Penaeidae demonstrated in this study, its conventional classification as a family can scarcely be sustained. Penaeidae can be maintained either by synonymizing it with Sicyoniidae, or raising the three penaeid tribes to the familial rank. The two major clades in our results correspond to the two-family scheme of Burkenroad (1983) with only Aristeidae and Penaeidae. However, the reciprocal monophyly of the three tribes demonstrated in the present study merits their recognition as distinct taxa. The levels of genetic divergence among the tribes and Sicyoniidae are comparable to those among Aristeidae, Benthesicymidae and Solenoceridae, and the evolutionary histories of these tribes are estimated to be longer than these four recognized families of Penaeoidea. Therefore, the tribes in Penaeidae warrant at least the same taxonomic rank as the latter. To maintain Sicyoniidae, and even Solenoceridae, the three tribes of Penaeidae should also be recognized as separate families, namely Penaeidae Rafinesque-Schmaltz, 1815, Parapenaeidae Ortmann, 1898 and Trachypenaeidae Burkenroad, 1983. Even if the two-family scheme of Burkenroad (1983) is followed, these three tribes will be

subfamilies equivalent to Solenocerinae, Aristeinae and Sicyoniinae, though synapomorphies of these three tribes have not yet been fully comprehended (see Burkenroad, 1983; Chan et al., 2008).

It is less clear whether Benthesicymidae warrants a family status. Crosnier (1985) treated it as a separate family from Aristeidae but several later studies did not follow his lead (e.g. Liu and Zhong, 1986; Dall et al., 1990; Hayashi, 1992; Chan, 1998). Pérez Farfante and Kensley (1997), however, revived the notion of Benthesicymidae as a separate family, and have been followed by Martin and Davis (2001). Unfortunately neither Crosnier (1985) nor his supporters have provided any detailed rationale for elevating benthesicymids into the family rank. The present results suggest that benthesicymids constitute a monophyletic group sister to aristeids. However, the sequence divergences between benthesicymids and aristeids (0.034 in NaK and 0.089 in PEPCK) are the lowest among all the major clades even including Burkenroad's (1983) penaeid tribes (tables 2.3 and 2.4). The level of divergence in NaK is lower than the values among family members except for Aristeidae and Benthesicymidae, while the divergence in PEPCK is lower than those among penaeid genera. Nevertheless, given the limited sampling of benthesicymids in this work, and as the two genera used have generally been considered to be very close, it would be more prudent to carry out a more comprehensive molecular study of these two families to determine if the family or even subfamily rank of benthesicymids can be justified.

Although the present molecular analyses have effectively resolved the familial and

tribal relationships in Penaeoidea, the relationships amongst the genera within each family and tribe remain mostly unresolved. Nevertheless, the results of this work provide strong genetic evidence to clarify the taxonomic status of several genera. The aristeid genus *Aristaeopsis*, containing only the monotypic species *A. edwardsiana*, has generally been regarded as a synonym of *Plesiopenaeus,* and it is only recently that a separate status has been proposed by Pérez Farfante and Kensley (1997). Our gene tree shows that *Aristaeopsis* is distinct from *Plesiopenaeus* but close to *Aristaemorpha* instead. Therefore, the generic status of *Aristaeopsis* is supported. On the other hand, *Hepomadus* and *Parahepomadus*, usually considered allies to *Aristaemorpha*, are genetically distinct from the latter. The molecular data confirm that the rare genus *Gordonella* is not a benthesicymid but belongs to Solenoceridae, and it is not close to *Haliporus* as suggested by Crosnier (1988). Moreover, our gene tree does not support at all the phylogenetic groupings of the solenocerid genera as proposed by Pérez Farfante (1977) and Kubo (1949). For Penaeidae, the splitting (i.e. polyphyly) of *Trachypenaeus s.l.* by Pérez Farfante and Kensley (1997) is strongly supported by our nuclear gene analysis, which in turn is consistent with results based on mitochondrial DNA (Chan et al., 2008).

2.4 Conclusion

The phylogenies of Penaeoidea inferred from morphology and molecular markers have been controversial. The present phylogenetic analysis using sequences of two nuclear protein-coding genes have yielded results, with high statistical support, which are largely consistent with the groupings of the morphology-inferred phylogeny above the genus level proposed by Burkenroad (1983). These have

provided new insights into the mode of diversification of the superfamily, age of divergence events and arguments for taxonomic revision in Penaeoidea. The paraphyly of Penaeidae and the large genetic divergence amongst the three penaeid tribes of Burkenroad (1983) and the other penaeoid families justify assigning the same taxonomic rank as Aristeidae, Solenoceridae and Sicyoniidae to the three tribes. The low genetic divergence between Aristeidae and Benthesicymidae suggests a re-evaluation of the family status of the latter. In showing that the penaeid-like lineage diverged earlier than the aristeid-like (and hence solenocerid-like) lineage, our results from molecular phylogenetic analyses are consistent with the evolutionary history revealed by fossil records and refute the evolutionary scenarios proposed by morphological analyses. The use of nuclear protein genes and more comprehensive taxon sampling of Sicyoniidae, Aristeidae and Solenoceridae than in the previous molecular studies have generated novel hypotheses for the evolution of genera or species in these families.

Chapter 3

Molecular phylogeny of genus *Penaeus sensu lato*

3.1 Introduction

Penaeus shrimps are a group of common marine shrimps with the highest economic value among all penaeids. After more than 10 years since Pérez Farfante and Kensley (1997) raised six subgenera of *Penaeus s.l.* shrimps to generic level, the debate on the legitimacy of the new classification scheme as well as its negative effects on fisheries and aquaculture was reignited recently (Dall, 2007; Flegel, 2007; Flegel, 2008; McLaughlin et al., 2008). New molecular phylogenetic studies have been urged upon to resolve the evolutionary relationships of the shrimps and justify the classification schemes. In additional to verifying taxonomic controversy, a better understanding of the evolutionary history of these shrimps can help discern alternative hypotheses on the temporal and spatial aspects of their origin and colonization pathways. Therefore the aims of this part of the study are to reconstruct the phylogeny of *Penaeus s.l.* species and their close allies (*Funchalia*, *Pelagopenaeus* and *Heteropenaeus*) using three nuclear protein coding genes (phosphoenolpyruvate carboxykinase (PEPCK), sodium-potassium ATPase α-subunit (NaK) and enolase) and two mitochondrial genes (16S and 12S rRNA) and to estimate their divergence time using the latest Bayesian relaxed clock approach (Drummond et al., 2006; Rutschmann, 2006).

3.2 Materials and methods

3.2.1 Taxon sampling

Shrimps were collected by trawling from the sea or by purchasing from local fish markets. Fifteen *Penaeus s.l.* species, together with three members of tribe Penaeini (*Funchalia* sp., *Pelagopenaeus balboae* and *Heteropenaeus longimanus*) were analyzed (table 3.1). Members from the other two tribes of Penaeidae, i.e. *Metapenaeopsis provocatoria longirostris*, *Penaeopsis eduardoi* and *Parapenaeus sextuberculatus* from Parapenaeini; and *Megokris pescadoreensis*, *Metapenaeus ensis and Trachysalambria starobogatovi* from Trachypenaeini were also analysed to provide reference of intergeneric divergence and to be used as outgroup taxa, together with a distant outgroup *Aristeus virilis* of family Aristeidae. Only one specimen per species was analyzed except for *Marsupenaeus japonicus* in which two genetically very distinct varieties (Tsoi et al., 2005) were analyzed. Species identification followed the keys of Crosnier (1978, 1988, 2003), Yu and Chan (1986), Liu and Zhong (1986), Pérez Farfante (1988), Dall et al. (1990), Pérez Farfante and Kensley (1997) and Chan (1998). Samples were either frozen at -70°C or preserved at 95% ethanol prior to DNA extraction.

3.2.2. DNA extraction, PCR and sequencing

Total genomic DNA was extracted from pleopod muscle using the commercial QIAamp Tissue Kit (QIAGEN). Primers for amplification of PEPCK and NaK were based on Tsang et al. (2008b) while the primer information for enolase, 16S and 12S was listed in table 3.2. Protocols of PCR amplification for PEPCK and NaK were same as that described in Chapter 2, section 2.2.2. Amplifications for enolase, 12S and 16S were carried out in a reaction mix containing 1-5 μl of template DNA, 1X

PCR reaction buffer, 3 mM $MgCl_2$, 200 μM dNTPs, 200 nM of each primer, 1.5 units of *Taq* polymerase (Amersham) and ddH_2O to a total volume of 50 μl. The PCR profile for these genes was as follows: 3 min at 94°C for initial denaturation, followed by 35 cycles of 30 s at 94°C, 30 s at 48-52°C (depending on individual samples), 1 min 30 s at 72°C with a final extension for 10 min at 72°C. The PCR products were purified using the QIAquick gel purification kit (QIAGEN) according to the manufacturer's instructions. The same sets of primers were used in sequencing reactions conducted by an Applied Biosystems 3100 automated sequencer using ABI Big-dye Ready-Reaction mix kit, following standard cycle sequencing protocol.

3.2.3. Phylogenetic analyses

Nucleotide sequences were aligned using CLUSTAL W (Thompson et al., 1994) using default parameters, manually adjusted, and confirmed by translating into amino acid sequences in case of protein-coding gene. The best-fit models of nucleotide substitution for the concatenated dataset and each gene were determined by Modeltest 3.7 (Posada and Crandall, 1998). Individual genes and the combined dataset were analysed under maximum likelihood (ML) using PhyML program (Guindon and Gascuel, 2003; Guindon et al., 2005; available at: http://atgc.lirmm.fr/phyml/). In ML analysis, two independent runs/analyses were performed with nodal support estimated from 1000 bootstrap (BP) pseudoreplicates. The concatenated data was partitioned by gene and separate model was assigned to each partition in the Bayesian inference (BI) analysis implemented in MrBayes v.3.12 (Ronquist and Huelsenbeck, 2003). Three independent runs were carried out with four differentially heated Metropolis coupled Monte Carlo Markov Chains for

Table 3.1. Classification and sampling locations of the species studied in the present study.

Tribe	Species	Sampling location
Penaeini	*Farfantepenaeus aztecus*	Gulf of Mexico
	Fenneropenaeus chinensis	Zhujiang estuary, China
	Fenneropenaeus merguiensis	Fish market, Hong Kong
	Funchalia sp.	Philippines
	Litopenaeus setiferus	Gulf of Mexico
	Litopenaeus vannamei	Fish market, Hong Kong
	Marsupenaeus japonicus Variety I	Singapore
	Marsupenaeus japonicus Variety II	Fish market, Hong Kong
	Melicertus canaliculatus	Taiwan
	Melicertus hathor	Isreal
	Melicertus kerathurus	Spain
	Heteropenaeus longimanus	Philippines
	Melicertus longistylus	New South Wales, Australia
	Melicertus plebejus	Queensland, Australia
	Melicertus latisulcatus	Taiwan
	Pelagopenaeus balboae	Indian Ocean
	Penaeus monodon	Fish market, Hong Kong
	Penaeus semiculatus	Indian Ocean
Outgroup		
Parapenaeini	*Metapenaeopsis provocatoria longirostris*	Taiwan
	Penaeopsis eduardoi	Taiwan
	Parapenaeus sextuberculatus	Taiwan
Trachypenaeini	*Megokris pescadoreensis*	Taiwan
	Metapenaeus ensis	Fish market, Hong Kong
	Trachysalambria starobogatovi	Natal, S. Africa
Aristeidae	*Aristeus virilis*	Taiwan

Table 3.2. Primer information for PCR amplification

Primer	Sequence (5' to 3')	Source
Enolase		
EF2	AGTTGGCTATGCAGGARTTYATGAT	Tsang et al. (in preparation)
ER2	ACCTGGTCGAATGGRTCYTC	Tsang et al. (in preparation)
16S		
AR	CGCCTGTTTATCAAAAACAT	Simon et al. (1994)
BR	CCGGTCTGAACTCAGATCACGT	Simon et al. (1994)
12S		
FB	GTGCCAGCAGCTGCGGTTA	Tsang et al. (submitted)
R2	CCTACTTTGTTACGACTTATCTC	Tsang et al. (submitted)

5,000,000 generations started from a random tree. Model parameters were estimated during the analysis. Chains were sampled every 500 generations and the trees before convergence were discarded as burn-in to ensure that analysis had stabilized (determined using Tracer v1.4, Rambaut and Drummond, 2004). Convergence was confirmed by monitoring likelihood values graphically. A 50% majority-rule consensus tree was constructed from the remaining trees to estimate posterior probabilities (PP).

Alternative phylogenetic hypotheses from previous morphological and molecular studies were tested using the Kishino–Hasegawa (KH) test (Kishino and Hasegawa, 1989) and Shimodaira–Hasegawa (SH) test (Shimodaira and Hasegawa, 1999) implemented in PAUP*. Alternative tree topologies were constructed using MacClade 3.0 (Maddison and Maddison, 1992) by rearranging the branches showing conflicting relationships between the ML tree and the a priori hypotheses. The tests were carried out with RELL optimization and 1000 bootstrap pseudoreplicates.

3.2.4. Divergence time estimation

BEAST v1.4.7 (Drummond and Rambaut, 2007) was used to estimate the divergence times of all nodes. Although fossils of *Penaeus* species have been discovered from different parts of the world, dated from the Jurassic to the lower Tertiary, the relationships of these fossils and the extant taxa have never been comprehensively studied. Therefore only one constraint could be applied with confidence that placed the divergence of *Penaeus s.l.* before the end of the Jurassic period (log normal prior, zero offset = 144 MYA, SD = 1).

The model for the gene-partitioned dataset was chosen by Modeltest 3.7. The uncorrelated lognormal relaxed molecular clock model with a Yule prior distribution for branching rates was employed. All of the Markov chain Monte Carlo analyses were run for 10 million generations with a burnin of one million generations and sampled every 1000 generations. The analyses were repeated to refine the tuning operators to improve efficiency using auto-optimize function in BEAST. Two separate runs were then combined and Tracer v1.4 was used to determine the effective sample size of each parameter and the node ages (Rambaut & Drummond 2004).

3.3 Results

3.3.1 Phylogenetic analyses

The aligned partial sequences of PEPCK gene included 527 bp, NaK gene included 580 bp, enolase gene included 351 bp, 16S rRNA gene included 516 bp and 12S rRNA gene included 432 bp, giving a total of 596 parsimony informative site in a total of 2410 bp (table 3.3). No introns or indels were observed in the three nuclear protein-coding genes. Ambiguous sites (double peaks in chromatograms), probably due to heterozygosity of individuals, were coded as ambiguous using the IUB symbols. Only sequences of PEPCK were slightly GC rich (43%). Sequences of NaK and enolase showed small AT bias (51% and 50.5% respectively) while those of 16S and 12S tend to be more AT bias (> 67%) (table 3.3). However there was no significant base heterogeneity across all codon positions in these genes (Chi-square $P > 0.05$) (table 3.3). The best-fit models selected by the Akaike Information

Criterion implemented in ModelTest for each of the genes and concatenated dataset are shown in table 3.4.

Tables 3.5-3.9 display the Kiruma 2-Parameter pairwise distance matrix of each gene while table 3.10 shows the summary of these distance information among penaeid shrimps. Mitochondrial rRNA genes were in general more variable than the nuclear protein-coding genes. The 12S rRNA gene showed the highest pairwise genetic distance (0.028-0.293), followed by 16S rRNA gene (0.017-0.209), enolase (0-0.189), PEPCK (0-0.156) and NaK appeared to be the most conserved gene (0-0.141).

The phylogenetic trees reconstructed for each gene using BI approach are shown in figs. 3.1-3.5. These trees revealed very different topologies but the overall posterior probabilities were low. The tree based on NaK found *Funchalia* and *Pelagopenaeus* nested within *Penaeus s.l.* while the 16S gene tree found *Heteropenaeus* grouped within *Penaeus s.l.*, but the supports were low in both case. Others gene tree supported the monophyly of *Penaeus s.l.* Nonetheless, they congruently showed that *Penaeus s.l.* contain two lineages: *Melicertus* + *Marsupenaeus* (henceforth called the *Melicertus* clade), and *Penaeus s.s.* + *Fenneropenaeus* + *Farfantepenaeus* + *Litopenaeus* (hereafter called the non-*Melicertus* clade). The tree topologies inferred from concatenated sequences using ML and BI approaches were identical and received high statistical supports in general. Here only the BI tree is presented (fig. 3.6) with support values for both BI and ML analyses. With the inclusion of related genera in Penaeini in the present analysis, the results strongly supported the

Table 3.3. Parsimony information of PEPCK, NaK, enolase, 16S and 12S.

Gene	No. of sites	No. of variable sites	No. of parsimony informative sites	% A/T	Chi-square test (*P*)
NaK					
nt1	194	23	17	43.6	1.000
nt2	193	11	6	61.7	1.000
nt3	193	123	90	47.6	1.000
All sites	580	157	113	51	1.000
PEPCK					
nt1	176	21	13	46.2	1.000
nt2	176	16	7	51.7	1.000
nt3	175	112	76	32	0.950
All sites	527	149	96	43.4	1.000
Enolase					
nt1	117	20	13	48	1.000
nt2	117	10	5	66.9	1.000
nt3	117	87	59	36.6	0.484
All sites	351	117	77	50.5	1.000
16S	432	162	127	67.7	1.000
12S	520	242	183	69.2	0.998
Overall:					
Nucleotide	2410	827	596	56.1	0.997

Table 3.4. Best fit model selected by the Akaike Information Criterion implemented in ModelTest.

	Best fit model	Detail settings
NaK	SYM+I+G	Base frequencies=equal; Nst=6; Rmat=1.3027, 4.3424, 2.5502, 1.2510, 11.2569; γ-shape parameter=0.8240; Proportion of invariable sites=0.5242
PEPCK	HKY+I+G	Base frequencies=0.2366, 0.3386, 0.2382; Nst=2; T Ratio=1.6305; γ-shape parameter=0.7615; Proportion of invariable sites=0.5427
Enolase	TIMef+I+G	Base frequencies=equal; Nst=6; Rmat=1.0000, 2.6534, 2.0217, 2.0217, 10.2385; γ-shape parameter=0.7559; Proportion of invariable sites=0.3882
16S	TrN+I+G	Base frequencies=0.3924, 0.0655, 0.1624; Nst=6; Rmat=1.0000, 6.7671, 1.0000, 1.0000, 18.6868; γ-shape parameter=0.5936; Proportion of invariable sites=0.5211
12S	GTR+I+G	Base frequencies=0.3708, 0.0613, 0.1697; Nst=6; Rmat=2.3307, 18.0149, 1.7877, 9.6768, 47.1304; γ-shape parameter=0.6113; Proportion of invariable sites=0.4248
Concatenated	GTR+I+G	Base frequencies=0.2824, 0.2039, 0.2227 ; Nst=6; Rmat=0.9509, 7.4897, 2.2855, 1.4633, 6.0664; γ-shape parameter=0.6630; Proportion of invariable sites=0.4813

Table 3.5. Table showing Kiruma-2-parameter pairwise divergence of NaK.

	Melicertus canaliculatus	*Melicertus hathor*	*Marsupenaeus japonicus I*	*Marsupenaeus japonicus II*	*Melicertus kerathurus*	*Melicertus latisulcatus*	*Melicertus longistylus*	*Melicertus plebejus*	*Farfantepenaeus aztecus*	*Litopenaeus setiferus*	*Litopenaeus vannamei*	*Fenneropenaeus chinensis*	*Fenneropenaeus merguiensis*	*Penaeus monodon*	*Penaeus semisulcatus*	*Funchalia* sp.	*Heteropenaeus longimanus*	*Pelagopenaeus balboae*	*Metapenaeopsis provocatoria longirostris*	*Penaeopsis eduardoi*	*Parapenaeus sextuberculatus*	*Metapenaeus ensis*	*Megokris pescadoreensis*	*Trachysalambria starobogatovi*
Melicertus canaliculatus																								
Melicertus hathor	0.014																							
Marsupenaeus japonicus I	0.011	0.008																						
Marsupenaeus japonicus II	0.009	0.005	0.002																					
Melicertus kerathurus	0.023	0.019	0.011	0.014																				
Melicertus latisulcatus	0.014	0.002	0.008	0.005	0.019																			
Melicertus longistylus	0.010	0.007	0.004	0.002	0.016	0.007																		
Melicertus plebejus	0.011	0.011	0.008	0.005	0.020	0.011	0.005																	
Farfantepenaeus aztecus	0.043	0.040	0.047	0.041	0.047	0.039	0.041	0.040																
Litopenaeus setiferus	0.041	0.041	0.045	0.039	0.045	0.041	0.039	0.038	0.010															
Litopenaeus vannamei	0.035	0.036	0.039	0.034	0.039	0.035	0.034	0.032	0.016	0.009														
Fenneropenaeus chinensis	0.043	0.045	0.047	0.041	0.048	0.043	0.041	0.044	0.032	0.030	0.028													
Fenneropenaeus merguiensis	0.047	0.049	0.049	0.045	0.052	0.047	0.043	0.050	0.037	0.035	0.034	0.009												
Penaeus monodon	0.045	0.045	0.047	0.041	0.047	0.043	0.041	0.044	0.028	0.027	0.025	0.010	0.012											
Penaeus semisulcatus	0.051	0.049	0.049	0.045	0.056	0.047	0.045	0.048	0.039	0.037	0.036	0.021	0.023	0.018										
Funchalia sp.	0.056	0.047	0.047	0.046	0.050	0.048	0.047	0.050	0.059	0.061	0.061	0.067	0.069	0.064	0.073									
Heteropenaeus longimanus	0.039	0.034	0.035	0.030	0.037	0.032	0.032	0.036	0.050	0.052	0.046	0.045	0.050	0.050	0.058	0.063								
Pelagopenaeus balboae	0.050	0.051	0.047	0.045	0.048	0.050	0.045	0.048	0.060	0.058	0.058	0.069	0.071	0.066	0.077	0.026	0.062							
Metapenaeopsis provocatoria longirostris	0.104	0.105	0.105	0.102	0.102	0.104	0.101	0.105	0.102	0.092	0.088	0.102	0.104	0.094	0.106	0.120	0.105	0.116						
Penaeopsis eduardoi	0.104	0.103	0.109	0.107	0.102	0.105	0.105	0.114	0.109	0.109	0.106	0.111	0.111	0.105	0.114	0.109	0.107	0.118	0.065					
Parapenaeus sextuberculatus	0.093	0.089	0.093	0.087	0.091	0.089	0.085	0.093	0.103	0.098	0.096	0.096	0.098	0.093	0.105	0.096	0.093	0.098	0.081	0.061				
Metapenaeus ensis	0.115	0.115	0.113	0.112	0.111	0.115	0.113	0.115	0.116	0.114	0.106	0.108	0.114	0.106	0.112	0.133	0.115	0.124	0.110	0.111	0.102			
Megokris pescadoreensis	0.114	0.113	0.105	0.110	0.119	0.117	0.110	0.111	0.114	0.114	0.112	0.123	0.131	0.121	0.127	0.120	0.115	0.126	0.123	0.113	0.100	0.107		
Trachysalambria starobogatovi	0.129	0.121	0.116	0.125	0.133	0.122	0.125	0.125	0.118	0.118	0.118	0.137	0.141	0.135	0.137	0.137	0.125	0.126	0.133	0.120	0.116	0.117	0.039	
Aristeus virilis	0.149	0.146	0.148	0.142	0.146	0.142	0.145	0.152	0.146	0.144	0.142	0.138	0.146	0.138	0.142	0.153	0.127	0.140	0.150	0.164	0.137	0.141	0.172	0.160

Table 3.6. Table showing Kiruma-2-parameter pairwise divergence of PEPCK.

	Melicertus canaliculatus	*Melicertus hathor*	*Marsupenaeus japonicus I*	*Marsupenaeus japonicus II*	*Melicertus kerathurus*	*Melicertus latisulcatus*	*Melicertus longistylus*	*Melicertus plebejus*	*Farfantepenaeus aztecus*	*Litopenaeus setiferus*	*Litopenaeus vannamei*	*Fenneropenaeus chinensis*	*Fenneropenaeus merguiensis*	*Penaeus monodon*	*Penaeus semisulcatus*	*Funchalia* sp.	*Heteropenaeus longimanus*	*Pelagopenaeus balboae*	*Metapenaeopsis provocatoria longirostris*	*Penaeopsis eduardoi*	*Parapenaeus sextuberculatus*	*Metapenaeus ensis*	*Megokris pescadoreensis*	*Trachysalambria starobogatovi*
Melicertus hathor	0.011																							
Marsupenaeus japonicus I	0.008	0.012																						
Marsupenaeus japonicus II	0.008	0.012	0.004																					
Melicertus kerathurus	0.031	0.035	0.031	0.029																				
Melicertus latisulcatus	0.013	0.000	0.013	0.013	0.037																			
Melicertus longistylus	0.013	0.013	0.010	0.010	0.025	0.015																		
Melicertus plebejus	0.012	0.016	0.012	0.012	0.040	0.018	0.014																	
Farfantepenaeus aztecus	0.060	0.050	0.060	0.060	0.066	0.051	0.051	0.061																
Litopenaeus setiferus	0.079	0.069	0.075	0.080	0.077	0.071	0.073	0.079	0.037															
Litopenaeus vannamei	0.064	0.054	0.059	0.064	0.066	0.055	0.053	0.065	0.017	0.025														
Fenneropenaeus chinensis	0.057	0.049	0.057	0.055	0.070	0.051	0.047	0.059	0.031	0.056	0.031													
Fenneropenaeus merguiensis	0.056	0.046	0.056	0.054	0.065	0.048	0.048	0.058	0.036	0.049	0.032	0.016												
Penaeus monodon	0.053	0.039	0.053	0.053	0.068	0.043	0.045	0.055	0.031	0.054	0.035	0.027	0.032											
Penaeus semisulcatus	0.042	0.036	0.046	0.046	0.054	0.038	0.038	0.047	0.028	0.049	0.032	0.022	0.018	0.022										
Funchalia sp.	0.059	0.055	0.064	0.064	0.076	0.055	0.062	0.065	0.066	0.084	0.072	0.064	0.061	0.062	0.048									
Heteropenaeus longimanus	0.060	0.058	0.060	0.060	0.065	0.060	0.054	0.062	0.076	0.083	0.078	0.078	0.072	0.067	0.054	0.069								
Pelagopenaeus balboae	0.056	0.046	0.056	0.056	0.064	0.048	0.054	0.057	0.061	0.068	0.065	0.065	0.057	0.056	0.049	0.040	0.052							
Metapenaeopsis provocatoria longirostris	0.124	0.107	0.124	0.117	0.115	0.108	0.117	0.127	0.113	0.123	0.111	0.109	0.095	0.098	0.084	0.111	0.119	0.089						
Penaeopsis eduardoi	0.102	0.098	0.110	0.104	0.113	0.099	0.106	0.109	0.106	0.112	0.106	0.107	0.100	0.091	0.074	0.097	0.095	0.079	0.063					
Parapenaeus sextuberculatus	0.099	0.098	0.108	0.102	0.111	0.099	0.104	0.107	0.109	0.121	0.111	0.107	0.107	0.096	0.080	0.095	0.099	0.083	0.072	0.023				
Metapenaeus ensis	0.136	0.132	0.138	0.131	0.138	0.134	0.138	0.140	0.139	0.137	0.136	0.127	0.124	0.134	0.113	0.136	0.134	0.119	0.122	0.102	0.108			
Megokris pescadoreensis	0.127	0.120	0.129	0.125	0.120	0.122	0.122	0.124	0.114	0.103	0.111	0.118	0.105	0.116	0.092	0.125	0.116	0.112	0.100	0.083	0.091	0.096		
Trachysalambria starobogatovi	0.151	0.145	0.156	0.149	0.147	0.147	0.149	0.148	0.143	0.129	0.140	0.143	0.131	0.135	0.120	0.142	0.142	0.136	0.104	0.093	0.100	0.117	0.043	
Aristeus virilis	0.108	0.106	0.115	0.111	0.104	0.108	0.104	0.116	0.106	0.107	0.095	0.111	0.101	0.109	0.082	0.113	0.110	0.103	0.102	0.091	0.091	0.124	0.107	0.115

Table 3.7. Table showing Kiruma-2-parameter pairwise divergence of enolase.

	Melicertus canaliculatus	*Melicertus hathor*	*Marsupenaeus japonicus I*	*Marsupenaeus japonicus II*	*Melicertus kerathurus*	*Melicertus latisulcatus*	*Melicertus longistylus*	*Melicertus plebejus*	*Farfantepenaeus aztecus*	*Litopenaeus setiferus*	*Litopenaeus vannamei*	*Fenneropenaeus chinensis*	*Fenneropenaeus merguiensis*	*Penaeus monodon*	*Penaeus semisulcatus*	*Funchalia* sp.	*Heteropenaeus longimanus*	*Pelagopenaeus balboae*	*Metapenaeopsis provocatoria longirostris*	*Penaeopsis eduardoi*	*Parapenaeus sextuberculatus*	*Metapenaeus ensis*	*Megokris pescadoreensis*	*Trachysalambria starobogatovi*
Melicertus hathor	0.014																							
Marsupenaeus japonicus I	0.006	0.014																						
Marsupenaeus japonicus II	0.006	0.015	0.000																					
Melicertus kerathurus	0.060	0.054	0.060	0.061																				
Melicertus latisulcatus	0.012	0.009	0.011	0.012	0.057																			
Melicertus longistylus	0.014	0.015	0.014	0.015	0.054	0.012																		
Melicertus plebejus	0.012	0.009	0.012	0.012	0.051	0.006	0.012																	
Farfantepenaeus aztecus	0.054	0.048	0.048	0.042	0.083	0.044	0.051	0.047																
Litopenaeus setiferus	0.057	0.051	0.057	0.051	0.083	0.048	0.054	0.051	0.017															
Litopenaeus vannamei	0.056	0.050	0.056	0.051	0.083	0.047	0.054	0.050	0.020	0.006														
Fenneropenaeus chinensis	0.056	0.050	0.048	0.042	0.092	0.047	0.054	0.050	0.020	0.029	0.029													
Fenneropenaeus merguiensis	0.047	0.041	0.044	0.042	0.076	0.038	0.044	0.041	0.023	0.023	0.023	0.014												
Penaeus monodon	0.056	0.050	0.056	0.048	0.086	0.047	0.054	0.050	0.017	0.017	0.023	0.014	0.017											
Penaeus semisulcatus	0.051	0.045	0.048	0.045	0.088	0.042	0.048	0.045	0.024	0.027	0.027	0.009	0.009	0.012										
Funchalia sp.	0.132	0.118	0.132	0.123	0.154	0.132	0.129	0.128	0.121	0.108	0.111	0.125	0.118	0.118	0.109									
Heteropenaeus longimanus	0.079	0.073	0.079	0.073	0.103	0.075	0.079	0.079	0.072	0.063	0.069	0.072	0.063	0.066	0.067	0.128								
Pelagopenaeus balboae	0.128	0.121	0.128	0.119	0.168	0.125	0.128	0.121	0.124	0.114	0.114	0.128	0.121	0.121	0.115	0.044	0.134							
Metapenaeopsis provocatoria longirostris	0.121	0.118	0.112	0.106	0.147	0.118	0.121	0.121	0.092	0.098	0.101	0.094	0.091	0.097	0.095	0.147	0.114	0.149						
Penaeopsis eduardoi	0.104	0.098	0.095	0.092	0.133	0.098	0.108	0.098	0.091	0.098	0.101	0.101	0.091	0.104	0.098	0.146	0.114	0.146	0.053					
Parapenaeus sextuberculatus	0.097	0.095	0.088	0.085	0.132	0.094	0.097	0.097	0.085	0.091	0.094	0.094	0.085	0.097	0.092	0.128	0.114	0.121	0.053	0.044				
Metapenaeus ensis	0.140	0.130	0.137	0.131	0.152	0.129	0.136	0.136	0.115	0.115	0.125	0.128	0.122	0.125	0.113	0.163	0.108	0.177	0.108	0.118	0.124			
Megokris pescadoreensis	0.144	0.133	0.151	0.142	0.126	0.140	0.147	0.140	0.115	0.119	0.122	0.139	0.132	0.125	0.120	0.157	0.125	0.178	0.118	0.125	0.138	0.076		
Trachysalambria starobogatovi	0.157	0.150	0.164	0.155	0.150	0.153	0.161	0.153	0.128	0.132	0.142	0.152	0.146	0.138	0.133	0.171	0.139	0.189	0.128	0.135	0.145	0.082	0.026	
Aristeus virilis	0.163	0.157	0.157	0.151	0.178	0.156	0.160	0.160	0.153	0.153	0.149	0.142	0.149	0.152	0.139	0.208	0.156	0.188	0.156	0.159	0.148	0.188	0.188	0.203

Table 3.8. Table showing Kiruma-2-parameter pairwise divergence of 12S.

	Melicertus canaliculatus	*Melicertus hathor*	*Marsupenaeus japonicus I*	*Marsupenaeus japonicus II*	*Melicertus kerathurus*	*Melicertus latisulcatus*	*Melicertus longistylus*	*Melicertus plebejus*	*Farfantepenaeus aztecus*	*Litopenaeus setiferus*	*Litopenaeus vannamei*	*Fenneropenaeus chinensis*	*Fenneropenaeus merguiensis*	*Penaeus monodon*	*Penaeus semisulcatus*	*Funchalia* sp.	*Heteropenaeus longimanus*	*Pelagopenaeus balboae*	*Metapenaeopsis provocatoria longirostris*	*Penaeopsis eduardoi*	*Parapenaeus sextuberculatus*	*Metapenaeus ensis*	*Megokris pescadoreensis*	*Trachysalambria starobogatovi*
Melicertus hathor	0.097																							
Marsupenaeus japonicus I	0.094	0.063																						
Marsupenaeus japonicus II	0.087	0.063	0.028																					
Melicertus kerathurus	0.104	0.095	0.097	0.090																				
Melicertus latisulcatus	0.085	0.044	0.054	0.056	0.097																			
Melicertus longistylus	0.090	0.086	0.093	0.093	0.094	0.070																		
Melicertus plebejus	0.092	0.048	0.065	0.068	0.085	0.039	0.074																	
Farfantepenaeus aztecus	0.129	0.121	0.119	0.124	0.141	0.114	0.146	0.117																
Litopenaeus setiferus	0.161	0.164	0.159	0.158	0.176	0.138	0.151	0.146	0.126															
Litopenaeus vannamei	0.156	0.136	0.136	0.138	0.156	0.112	0.151	0.136	0.105	0.082														
Fenneropenaeus chinensis	0.159	0.151	0.152	0.151	0.159	0.141	0.165	0.144	0.149	0.116	0.129													
Fenneropenaeus merguiensis	0.184	0.154	0.142	0.152	0.144	0.137	0.162	0.144	0.143	0.134	0.127	0.054												
Penaeus monodon	0.185	0.145	0.147	0.151	0.182	0.138	0.185	0.156	0.131	0.146	0.136	0.113	0.115											
Penaeus semisulcatus	0.157	0.141	0.139	0.139	0.136	0.127	0.151	0.149	0.135	0.125	0.132	0.094	0.092	0.110										
Funchalia sp.	0.184	0.182	0.159	0.158	0.169	0.161	0.203	0.166	0.165	0.177	0.159	0.159	0.151	0.150	0.163									
Heteropenaeus longimanus	0.193	0.176	0.163	0.163	0.181	0.158	0.187	0.153	0.196	0.182	0.193	0.163	0.171	0.177	0.173	0.157								
Pelagopenaeus balboae	0.209	0.196	0.175	0.172	0.199	0.174	0.199	0.180	0.167	0.195	0.174	0.177	0.175	0.167	0.189	0.096	0.182							
Metapenaeopsis provocatoria longirostris	0.224	0.215	0.230	0.227	0.234	0.221	0.244	0.226	0.197	0.208	0.200	0.204	0.227	0.191	0.200	0.202	0.227	0.207						
Penaeopsis eduardoi	0.190	0.183	0.182	0.179	0.170	0.185	0.207	0.198	0.169	0.187	0.168	0.171	0.172	0.171	0.172	0.156	0.192	0.159	0.126					
Parapenaeus sextuberculatus	0.205	0.179	0.201	0.195	0.193	0.200	0.198	0.200	0.185	0.187	0.192	0.192	0.198	0.189	0.185	0.177	0.203	0.167	0.127	0.099				
Metapenaeus ensis	0.201	0.160	0.175	0.194	0.195	0.176	0.200	0.170	0.164	0.204	0.192	0.198	0.180	0.176	0.180	0.231	0.238	0.226	0.175	0.151	0.152			
Megokris pescadoreensis	0.258	0.238	0.249	0.252	0.258	0.235	0.261	0.241	0.208	0.238	0.216	0.233	0.225	0.211	0.223	0.212	0.263	0.225	0.229	0.204	0.213	0.197		
Trachysalambria starobogatovi	0.262	0.268	0.298	0.282	0.296	0.265	0.280	0.268	0.250	0.256	0.233	0.270	0.280	0.266	0.269	0.259	0.287	0.268	0.221	0.232	0.249	0.239	0.165	
Aristeus virilis	0.237	0.209	0.217	0.220	0.248	0.226	0.243	0.229	0.230	0.224	0.228	0.193	0.205	0.212	0.211	0.230	0.222	0.236	0.201	0.178	0.167	0.199	0.246	0.287

Table 3.9. Table showing Kiruma-2-parameter pairwise divergence of 16S.

	Melicertus canaliculatus	*Melicertus hathor*	*Marsupenaeus japonicus I*	*Marsupenaeus japonicus II*	*Melicertus kerathurus*	*Melicertus latisulcatus*	*Melicertus longistylus*	*Melicertus plebejus*	*Farfantepenaeus aztecus*	*Litopenaeus setiferus*	*Litopenaeus vannamei*	*Fenneropenaeus chinensis*	*Fenneropenaeus merguiensis*	*Penaeus monodon*	*Penaeus semisulcatus*	*Funchalia* sp.	*Heteropenaeus longimanus*	*Pelagopenaeus balboae*	*Metapenaeopsis provocatoria longirostris*	*Penaeopsis eduardoi*	*Parapenaeus sextuberculatus*	*Metapenaeus ensis*	*Megokris pescadoreensis*	*Trachysalambria starobogatovi*
Melicertus hathor	0.069																							
Marsupenaeus japonicus I	0.060	0.055																						
Marsupenaeus japonicus II	0.042	0.054	0.017																					
Melicertus kerathurus	0.049	0.071	0.071	0.050																				
Melicertus latisulcatus	0.059	0.045	0.068	0.061	0.073																			
Melicertus longistylus	0.054	0.058	0.047	0.042	0.054	0.052																		
Melicertus plebejus	0.057	0.050	0.068	0.058	0.054	0.037	0.027																	
Farfantepenaeus aztecus	0.124	0.140	0.125	0.117	0.115	0.138	0.116	0.133																
Litopenaeus setiferus	0.157	0.178	0.162	0.160	0.163	0.161	0.168	0.175	0.126															
Litopenaeus vannamei	0.124	0.137	0.127	0.125	0.118	0.126	0.135	0.135	0.085	0.086														
Fenneropenaeus chinensis	0.127	0.126	0.113	0.109	0.112	0.118	0.127	0.136	0.124	0.122	0.118													
Fenneropenaeus merguiensis	0.110	0.113	0.103	0.095	0.096	0.108	0.110	0.127	0.102	0.122	0.102	0.037												
Penaeus monodon	0.112	0.124	0.111	0.098	0.093	0.124	0.104	0.118	0.121	0.140	0.118	0.086	0.075											
Penaeus semisulcatus	0.090	0.106	0.105	0.092	0.085	0.096	0.088	0.090	0.105	0.135	0.107	0.089	0.081	0.088										
Funchalia sp.	0.112	0.111	0.114	0.096	0.092	0.117	0.106	0.120	0.139	0.171	0.136	0.119	0.108	0.094	0.125									
Heteropenaeus longimanus	0.130	0.157	0.149	0.123	0.124	0.142	0.133	0.133	0.177	0.209	0.183	0.170	0.171	0.147	0.146	0.143								
Pelagopenaeus balboae	0.123	0.140	0.135	0.139	0.109	0.129	0.138	0.141	0.152	0.168	0.142	0.134	0.123	0.114	0.128	0.052	0.159							
Metapenaeopsis provocatoria longirostris	0.167	0.174	0.166	0.155	0.152	0.165	0.167	0.171	0.173	0.180	0.161	0.161	0.146	0.157	0.167	0.146	0.205	0.161						
Penaeopsis eduardoi	0.118	0.131	0.133	0.122	0.112	0.127	0.121	0.130	0.132	0.120	0.115	0.132	0.115	0.118	0.115	0.097	0.153	0.113	0.095					
Parapenaeus sextuberculatus	0.116	0.112	0.137	0.117	0.105	0.119	0.130	0.124	0.139	0.127	0.121	0.133	0.119	0.118	0.107	0.092	0.154	0.100	0.104	0.042				
Metapenaeus ensis	0.160	0.181	0.191	0.175	0.152	0.172	0.163	0.166	0.203	0.202	0.182	0.190	0.172	0.166	0.160	0.160	0.200	0.169	0.136	0.133	0.110			
Megokris pescadoreensis	0.133	0.169	0.154	0.153	0.125	0.154	0.142	0.157	0.171	0.150	0.156	0.150	0.136	0.142	0.136	0.128	0.166	0.142	0.112	0.088	0.100	0.130		
Trachysalambria starobogatovi	0.117	0.147	0.142	0.131	0.126	0.120	0.117	0.131	0.175	0.183	0.163	0.152	0.149	0.146	0.125	0.132	0.170	0.167	0.131	0.098	0.090	0.138	0.053	
Aristeus virilis	0.152	0.168	0.170	0.155	0.149	0.170	0.149	0.164	0.194	0.230	0.200	0.200	0.190	0.191	0.169	0.183	0.198	0.185	0.171	0.163	0.155	0.152	0.162	0.160

Table 3.10. Summary of pairwise distance of NaK, PEPCK, enolase, 16S and 12S, showing the range and average of distance in different grouping. * included *Heteropenaeus longimanus*, *Funchalia* sp. and *Pelagopenaeus balboae*.

	NaK	PEPCK	Enolase	16S	12S
Within *Melicertus* clade	0.002-0.023 (0.01)	0-0.04 (0.017)	0-0.061 (0.024)	0.017-0.073 (0.054)	0.028-0.097 (0.072)
Within Non-*Melicertus* clade	0.009-0.039 (0.025)	0.016-0.056 (0.032)	0.006-0.029 (0.019)	0.037-0.14 (0.103)	0.054-0.146 (0.114)
Between *Melicertus* and Non-*Melicertus* clade	0.032-0.056 (0.043)	0.036-0.08 (0.057)	0.017-0.092 (0.05)	0.085-0.178 (0.122)	0.105-0.185 (0.144)
Within Parapenaeini	0.061-0.081 (0.069)	0.023-0.072 (0.053)	0.044-0.044 (0.044)	0.042-0.104 (0.08)	0.099-0.099 (0.099)
Within Trachypenaeini	0.039-0.117 (0.087)	0.043-0.117 (0.085)	0.026-0.026 (0.026)	0.053-0.138 (0.107)	0.165-0.165 (0.165)
Within Penaeini (excluding *Penaeus s.l.*) *	0.026-0.063 (0.05)	0.04-0.069 (0.054)	0.134-0.134 (0.134)	0.052-0.159 (0.118)	0.182-0.182 (0.182)
Between *Penaeus s.s.* + *Fenneropenaeus* and *Farfantepenaeus* + *Litopenaeus*	0.025-0.039 (0.032)	0.028-0.056 (0.039)	0.009-0.029 (0.022)	0.102-0.14 (0.118)	0.054-0.146 (0.119)

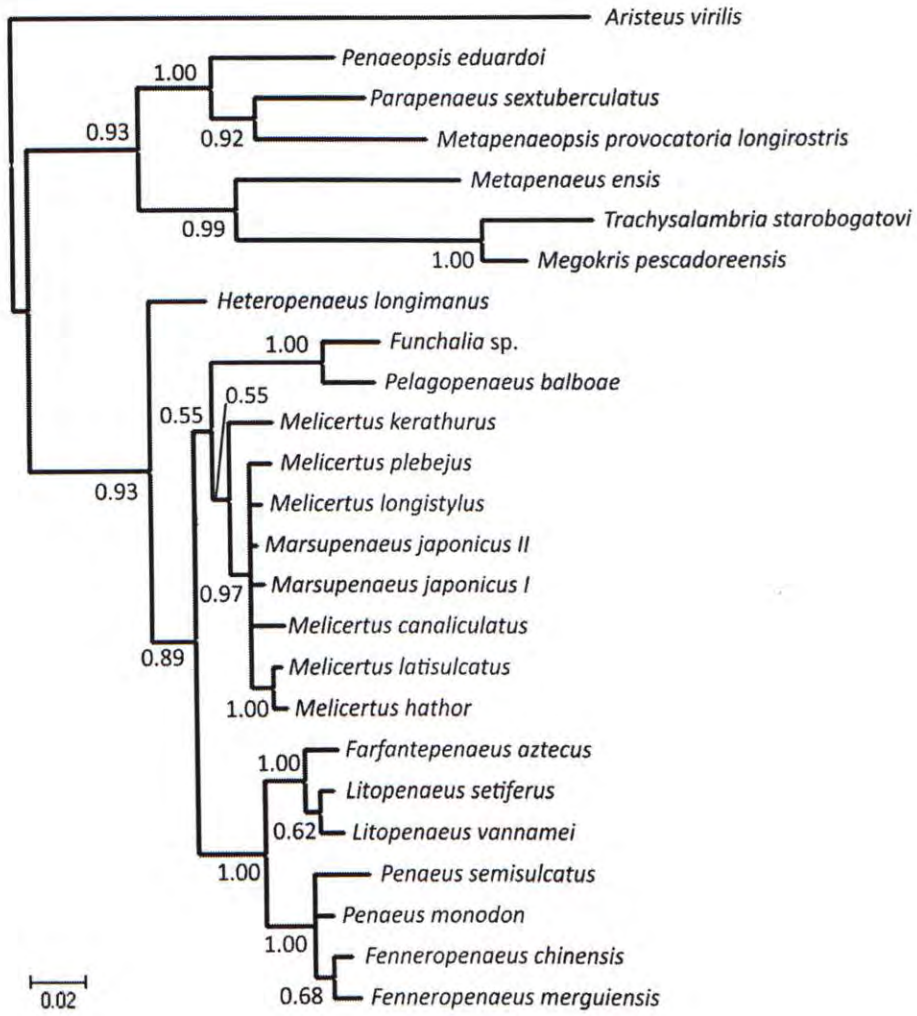

Figure 3.1. Bayesian inference tree based on NaK gene. Numbers near nodes indicate posterior probability values from BI. Values below 0.5 are not shown.

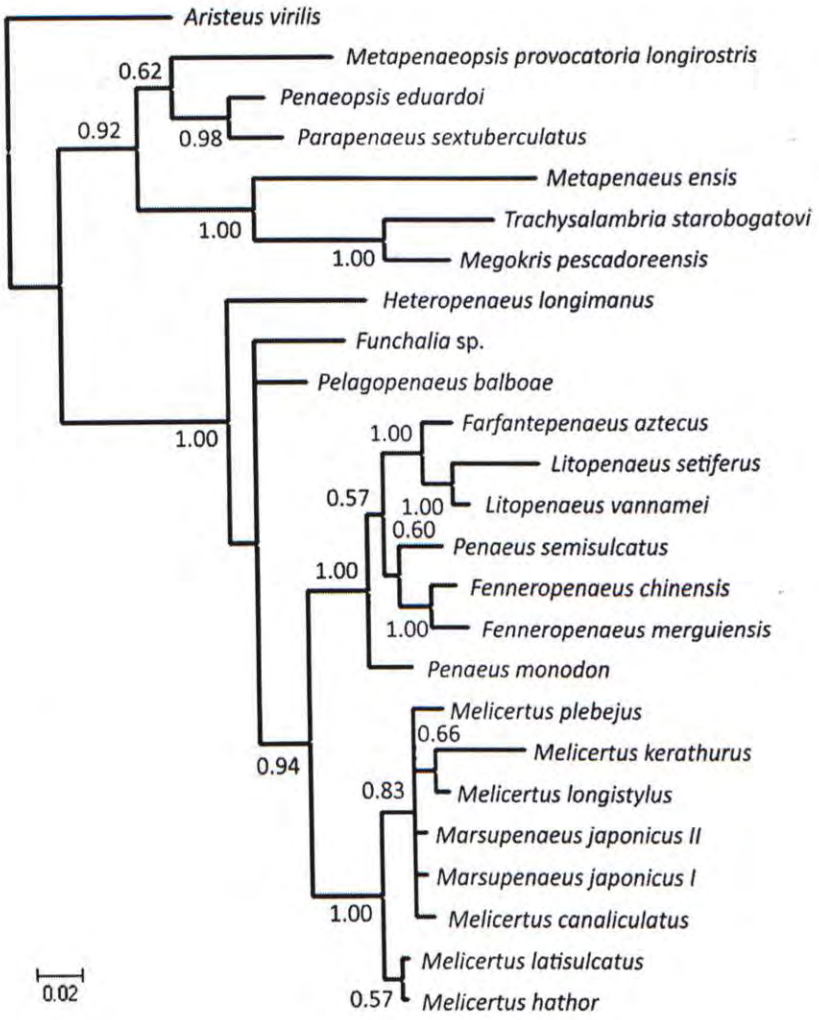

Figure 3.2. Bayesian inference tree based on PEPCK gene. Numbers near nodes indicate posterior probability values from BI. Values below 0.5 are not shown.

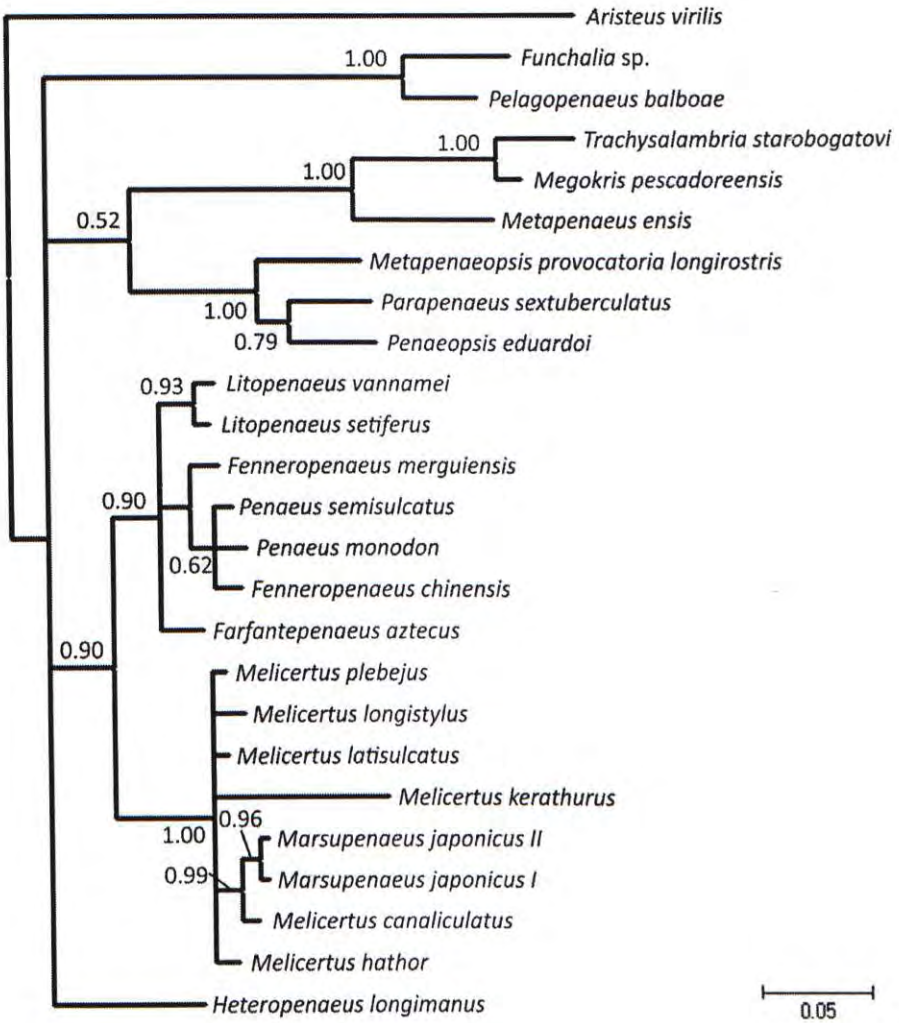

Figure 3.3. Bayesian inference tree based on enolase gene. Numbers near nodes indicate posterior probability values from BI. Values below 0.5 are not shown.

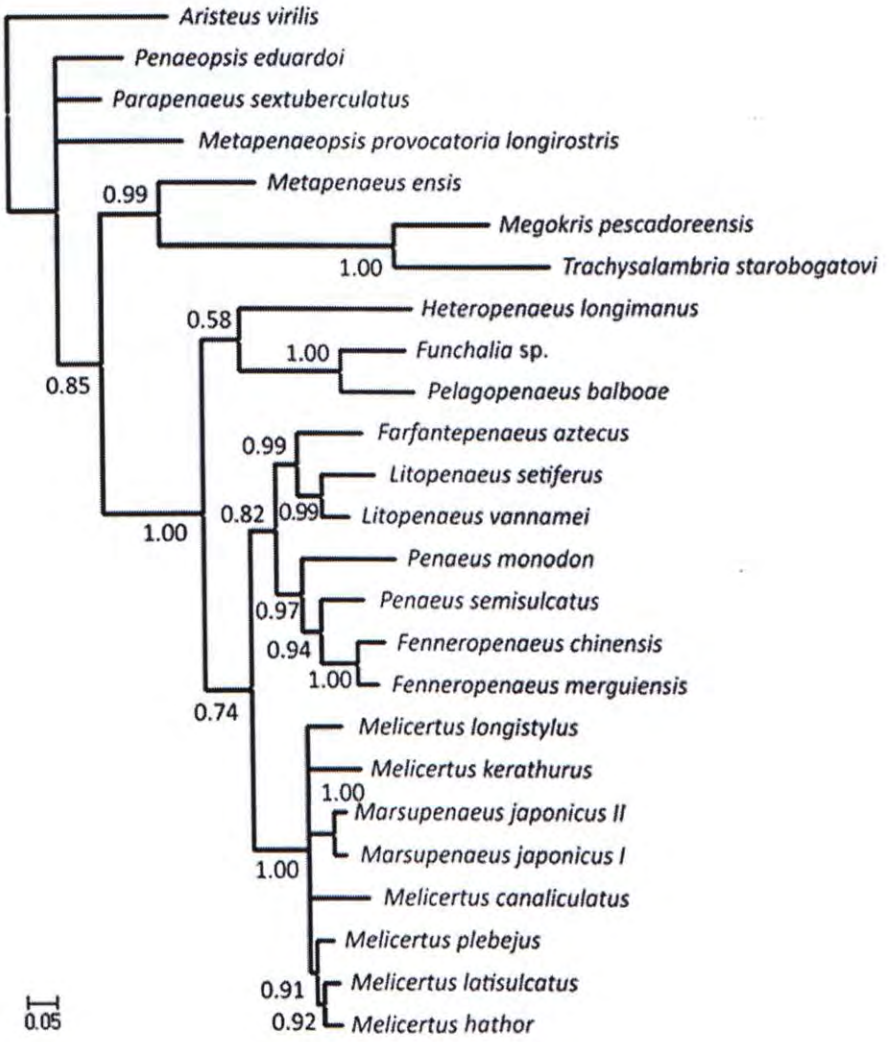

Figure 3.4. Bayesian inference tree based on mitochondrial 12S rRNA gene. Numbers near nodes indicate posterior probability values from BI. Values below 0.5 are not shown.

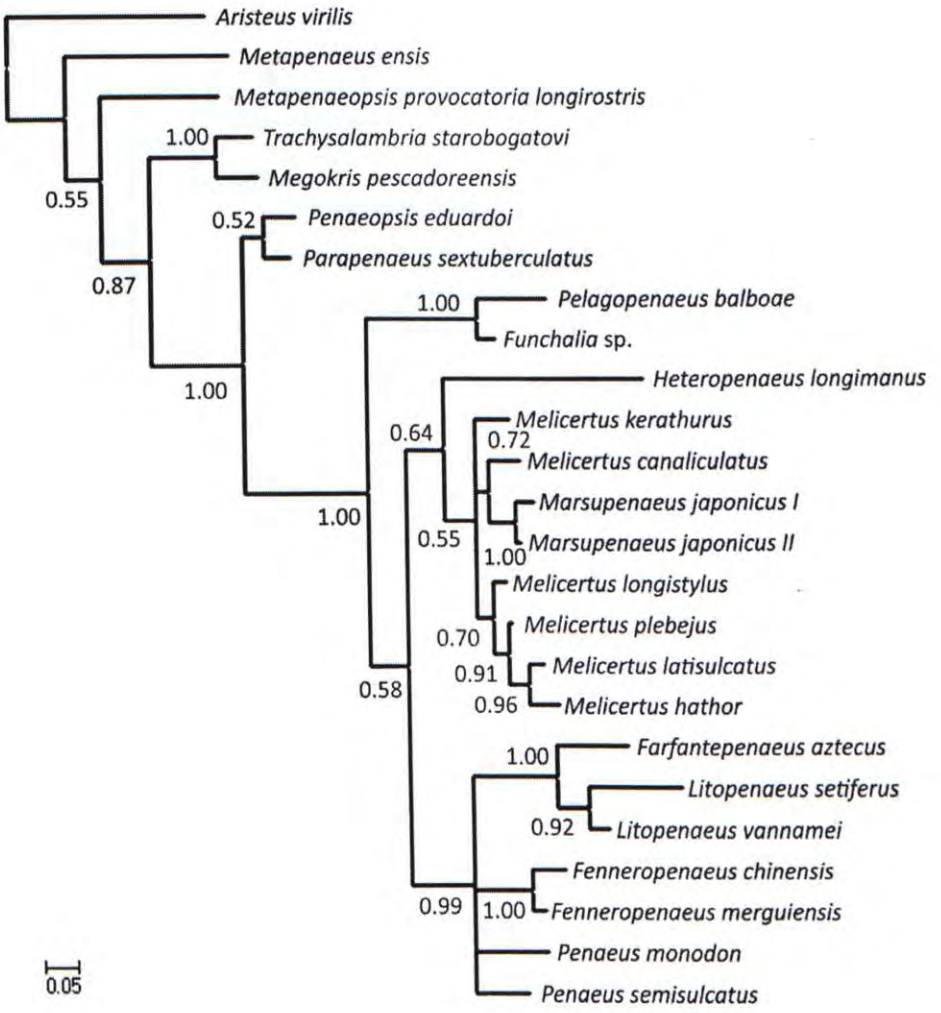

Figure 3.5. Bayesian inference tree based on mitochondrial 16S rRNA gene. Numbers near nodes indicate posterior probability values from BI. Values below 0.5 are not shown.

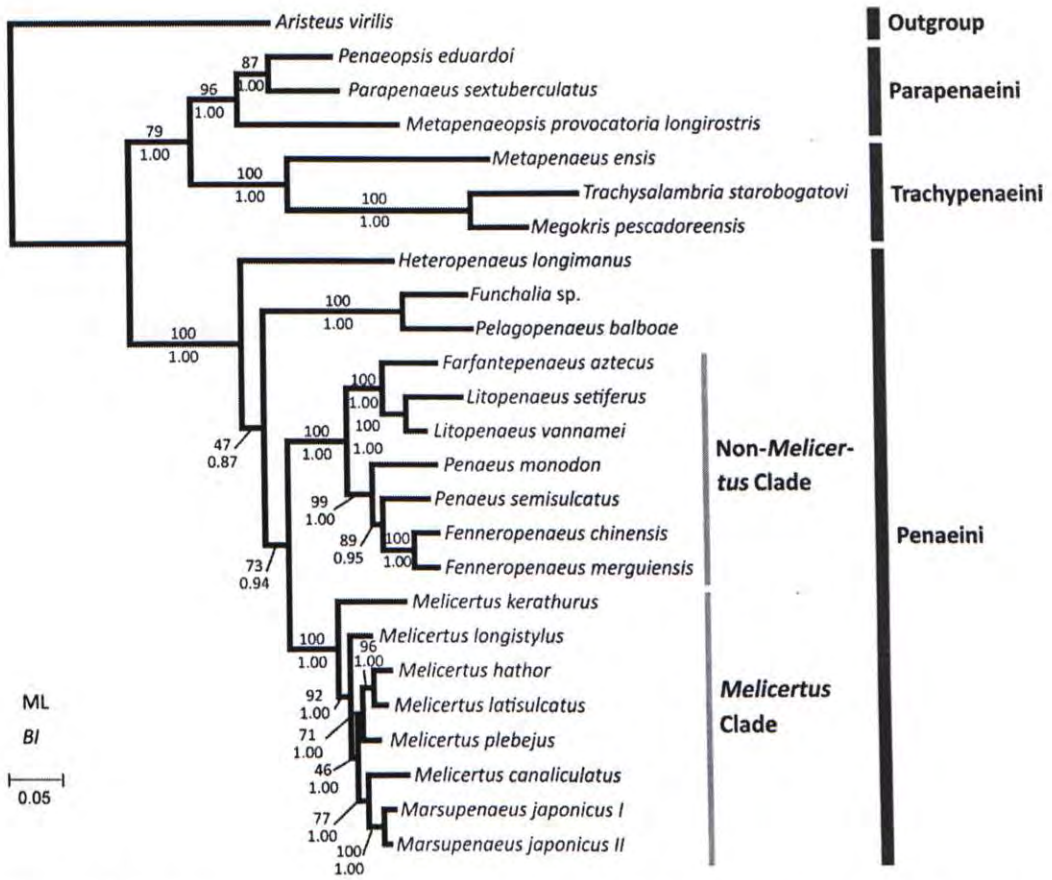

Figure 3.6. Bayesian inference tree from combined sequences analysis under the best-fitting model of each gene. Numbers above branches indicate bootstrap values from maximum likelihood while posterior probabilities from BI are indicated below branches. Values below 50 are not shown.

monophyly of *Penaeus s.l.* The molecular analyses also provided robust evidences for the monophyly of *Fenneropenaeus* and *Litopenaeus*, the latter two of which have always been found as sister taxa (Maggioni et al., 2001, Lavery et al., 2004). *Penaeus s.s* was found paraphyletic, with *Fenneropenaeus* nested within. *Marsupenaeus* grouped within *Melicertus* and hence challenged its monophyly. SH and KH tests strongly supported that *Melicertus* is not monophyletic ($P < 0.05$ in both tests) but the hypothesis of monophyly of *Penaeus s.s.* cannot be rejected. Phylogenetic analyses robustly supported that the genus *Penaeus s.l.* contains the two distinct *Melicertus* and non-*Melicertus* clades. The average genetic distances between these two clades ranged from 0.043 in NaK to 0.147 in 12S (table 3.5-3.9). This level of divergence was comparable to that between the remaining three genera of Penaeini, i.e., *Heteropenaeus longimanus*, *Funchalia* sp. and *Pelagopenaeus balboae* (0.05-0.147). The divergence was even slightly higher than the intergeneric distance in the outgroup Parapenaeini (0.05-0.117).

3.3.1.1 Melicertus *clade*

The evolutionary relationships within the *Melicertus* clade were clearly elucidated in our molecular analyses. Our analyses placed *M. kerathurus* at the basal position while *M. longistylus* was also found distantly related to the rest of the clade. *M. hathor*, which is sometimes recognized as a western subspecies of *M. latisculatus* (Miquel, 1984), was shown to be closely associated with the latter species (genetic distance ranged from 0 in PEPCK to 0.087 in 12S, table 3.5-3.9), and they together were sister to *M. plebejus*. A tight affinity of *M. canaliculatus* to *Marsupenaeus japonicus* was also suggested basing on our molecular data, and this implied that

Melicertus was paraphyletic. The association of these two species agreed with the fact that they share high morphological similarity except in their thelycum.

*3.3.1.2 Non-*Melicertus *clade*

The non-*Melicertus* clade diverged into two rather distinct lineages (average divergence as high as 0.134 in 12S, table 3.8). In one lineage, the two Western Hemisphere taxa, *Farfantepenaeus* and *Litopenaeus*, grouped together with high support. With only one sample of *Farfantepenaeus* species and two *Litopenaeus* samples, this study cannot provide substantial evidence to prove whether or not these two taxa are reciprocally monophyletic. However it should be noted that previous studies based on mitochondrial gene sequences had already presented strong supports for their monophyly (Maggioni et al., 2001; Lavery et al., 2004). In the other lineage, *Penaeus s.s.* were found to be paraphyletic and basal to *Fenneropenaeus* although the sister relationship between *Penaeus semiculatus* and the two *Fenneropenaeus* species only received moderate support. Our analyses showed that *Penaeus monodon* occupied the basal position in this *Penaeus s.s.* + *Fenneropenaeus* lineage.

3.3.2 Divergence time estimations

The effective sample sizes of all parameters were well above optimal level (data not shown) in each 10-million-generation run implemented in BEAST v1.4.7, ensuring that the chains of the analyses were run long enough. The divergence times estimated are shown in fig. 3.7 with 95% credibility interval and the posterior mean age indicated. The results showed that Penaeini diverged from other penaeid tribes at

about 304 MYA and in 198 MYA *Heteropenaeus* was established. At about 174 MYA *Funchalia* and *Pelagopenaeus* separated from the ancestors of *Penaeus s.l*, which in the early Cretaceous (146 MYA) divided into the *Melicertus* and non-*Melicertus* lineages. The non-*Melicertus* has a longer evolutionary history. The Western Hemisphere *Penaeus* shrimps diverged from their Indo-West Pacific relatives around 94 MYA and these American shrimps further diversified at about the beginning of the Tertiary epoch (59 MYA). Concordantly, the times of diversification of *Melicertus* and the *Penaeus s.s.* + *Fenneropenaeus* clade were approximated at the K-T boundary (62 and 66 MYA respectively). Most speciation events within *Melicertus* occurred during the Paleocene.

3.4 Discussion

3.4.1 Phylogeny and taxonomic implications

The present study utilized the most extensive genetic dataset to scrutinize the phylogeny of *Penaeus s.l.* thus far, and the resulting tree received much stronger statistical support than previous studies. The addition of nuclear markers in the analyses improves the credibility of the reconstructed phylogeny. The fact that the phylogenetic relationships inferred from this study employing nuclear markers are congruent with those based on mitochondrial COI and 16S genes (Lavery et al., 2004) may imply that the suggested phylogeny can reliably reflect the evolutionary history of *Penaeus s.l.* taxa, despite its contradictions to the conventional ones based on morphology (Burkenroad, 1934; Kubo, 1949; Pérez Farfante, 1969; Timizi, 1971; Burukovsky, 1972; Pérez Farfante and Kensley, 1997). Our results challenged the traditional morphology-based phylogeny in several ways; nonetheless the

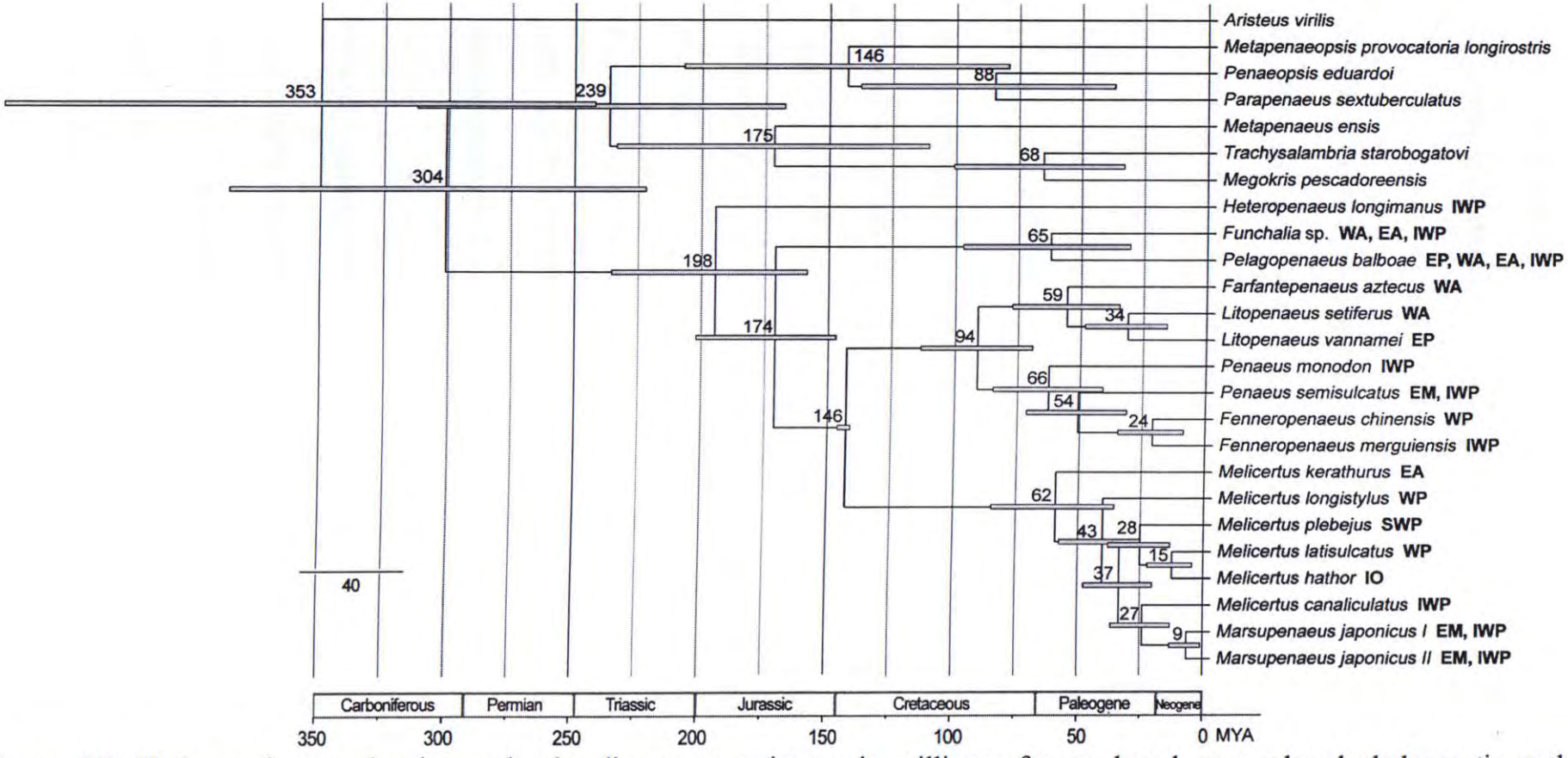

Figure 3.7. Phylogenetic tree showing molecular divergence estimates in millions of years based on a relaxed phylogenetic analysis of concatenated sequence data with grey bars showing 95% credibility intervals and posterior mean age adjacent to each node. The bolded letters on the right of species names denote the distribution ranges: EP=East Pacific; WA=West Atlantic; EA=East Atlantic; IO=Indian Ocean; IWP=Indo-West Pacific, SWP=Southwest Pacific (Oceania); WP=West Pacific.

morphological characters employed previously may not be synapomorphic and hence may not be phylogenetically informative. The importance of these characters for phylogenetic inference of shrimps may have to be reconsidered.

Firstly, it was strongly supported by both the current study and Lavery et al. (2004) that *Penaeus s.l.* diverged to form the *Melicertus* and non-*Melicertus* clades. This division, however, opposed Burkenroad (1934)'s view that grouped together shrimps with gastrofrontal groove in carapace. Based on this scheme, *Farfantepenaeus*, with the presence of straight gastrofrontal groove, would be more closely related with *Melicertus* and *Marsupenaeus* whose gastrofrontal groove curve anterodorsally at the posterior end, instead of grouping in the non-*Melicertus* clade with species without the gastrofrontal groove. However, Pérez Farfante and Kensley (1997) also indicated that spines are present on the telson of members of the *Melicertus* clade (except in *M. canaliculatus*) and the basal taxa of tribe Penaeini (i.e. *Heteropenaeus*, *Funchalia* and *Pelagopenaeus*) but they are absent in the non-*Melicertus* lineage. Possession of telson spines may be an ancestral character that is independently lost in the non-*Melicertus* lineage and also *M. canaliculatus* which is one of the most derived species in the *Melicertus* clade.

Secondly, among the "non-grooved" *Penaeus* shrimps, Kubo (1949) proposed a close affinity between *Litopenaeus* and *Penaeus s.s.* as they both have hepatic ridge on their carapace while *Fenneropenaeus* does not. Yet, preceding (Lavery et al., 2004; Voloch et al., 2005) and the current molecular studies congruently suggest that *Litopenaeus* is more related to *Farfantepenaeus* while *Fenneropenaeus* is nested

within *Penaeus* s.s with robust support (fig. 3.6). Although the morphology of the non-*Melicertus* clade taxa varies significantly and can allow easy distinction of the subgenera (such as the presence of special semi-open thelycum and short ventral costae in *Litopenaeus*, and the absence of hepatic ridge in *Fenneropenaeus*), no single or a combination of morphological characters described in the monograph by Pérez Farfante and Kensley (1997) can clearly separate the two lineages within this clade. A possible way to distinguish the two lineages is by their geographical distributions: *Fenneropenaeus* and *Penaeus s.s.* inhabit the Indo-West Pacific region whereas *Litopenaeus* and *Farfantepenaeus* live in the Western Hemisphere.

Thirdly, the separation of the single-species taxon *Marsupenaeus* from *Melicertus* was not supported by the current study as well as Lavery et al. (2004) which concordantly found the former nested within *Melicertus* and associated with *Melicertus canaliculatus* with rather low genetic divergence (table 3.5-3.9) that is only comparable to species level divergence. Despite having very peculiar tube-like thelycum, this unique trait of *Marsupenaeus japonicus* may be autapomorphic and not phylogenetically informative.

Previous molecular phylogenetic studies of Penaeidae based on 16S (Chan et al., 2008) and the two nuclear protein-coding genes in the previous part of this study (see Chapter 2) suggested that *Heteropenaeus* or *Funchalia* may nest within *Penaeus s.l.*, though the statistical supports for these phylogenetic hypotheses were weak. Such results challenged the monophyly of *Penaeus s.l.*, suggesting that it would not be "natural" to keep *Penaeus s.l.* as an intact taxonomic unit. However, by

incorporating more genetic data and extensive sampling that include all Penaeini genera, together with more outgroup taxa from Penaeoidea, the current study supports the monophyly of *Penaeus s.l.*, implying that it is not necessary to divide this genus. If we wish to have a taxonomy that truly reflects evolutionary relationships of the *Penaeus s.l.* shrimps, the classification proposed by Pérez Farfante and Kensley (1997) would certainly be refuted (as *Melicertus* and *Penaeus s.s.* are confirmed to be paraphyletic), and here four schemes can be proposed such that each taxonomic unit is monophyletic with strong support in this molecular study (see below). However before taxonomy can be revised, especially when the taxonomic ranking is involved, it is necessary to also consider the genetic divergence among the groups and if there are any diagnostic characters. Having the genetic data from 6 different genera of Trachypenaeini and Parapenaeini for reference, hereafter I will discuss the legitimacy of different revision schemes based on genetic distances among the groups and their morphology.

The first scheme is to group them back as one genus, *Penaeus*. Combining all 28 species into a single genus would certainly render this taxon very diverse. Although the level of genetic and morphological divergence in this single genus would be high when compared to most other Penaeidae and Penaeoidea genera, there appears no contradiction to taxonomic rules to retain the old classification scheme.

The second to fourth schemes are to divide *Penaeus* s.l. into two to four units/genera in the following ways:

Scheme II: Division into four units: (1) *Melicertus* + *Marsupenaeus*, (2) *Penaeus* +

Fenneropenaeus, (3) *Farfantepenaeus* and (4) *Litopenaeus*.

Scheme III: Division into three units: (1) *Melicertus* + *Marsupenaeus*, (2) *Penaeus* + *Fenneropenaeus* and (3) *Farfantepenaeus* + *Litopenaeus*.

Scheme IV: Division into two units: (1) *Melicertus* + *Marsupenaeus*, (2) *Penaeus* + *Fenneropenaeus*, *Farfantepenaeus* and *Litopenaeus*.

Although the genetic distances among all units in the three schemes proposed are high and comparable to the intergeneric level in Parapenaeini, it is difficult to define the groups morphologically. Unit (1), i.e. the *Melicertus* clade, in all of the schemes is distantly related to the rest of the species and the genetic distances between this unit and the others are high and comparable to the intergeneric level in other penaeid shrimps. Morphologically, although all non-*Melicertus* shrimps lack telson spines that are commonly found in unit (1) species, the telson of *Melicertus canaliculatus* is also unarmed, making this character not synapomorphic. These three schemes are therefore not supported unless future morphological studies of the *Penaeus s.l.* species could identify diagnostic, synapomorphic characters among these groups. Meanwhile, it would be inappropriate to adopt the classification scheme of Pérez Farfante and Kensley (1997). The old classification scheme that assigns all 28 species into a single *Penaeus* genus is more proper.

The taxonomic status of *Melicertus hathor* has also been controversial since Pérez Farfante and Kensley (1997) assigned a species rank to this shrimp which was sometimes regarded as a western subspecies of *M. latisulcatus* (Miquel, 1984). Most taxonomists, however, tend not to recognize this species or subspecies (e.g., Chan, 1998; Dall et al., 1990; Hayashi, 1992; Holthuis and Miquel, 1984). The genetic

divergence of 16S, 12S and enolase between these two species are found to be higher than some of the interspecific divergence in this study (tables 3.5-3.9). Hence our results support the recognition of *M. hathor* as a distinct species.

3.4.2 Divergence time and phylogeography

Understanding the time of diversification can allow correlations between past geological changes and organisms' evolutionary history. This study provides the first divergence time estimation of the *Penaeus s.l.* species, the knowledge of which can help discern alternative hypothesis regarding the origin and colonization pathways of this genus.

The northwest Tethys Sea (southern Europe) appears to be a reasonable site of origin of the *Penaeus s.l.* species. Most of the Jurassic and Cretaceous *Penaeus* fossils were discovered in southern Europe while fossils uncovered in India were dated to the late Tertiary (Glaessner, 1969). These suggest that ancestors of *Penaeus s.l.* were established in shallow-waters of the northwest Tethys Sea around the late Jurassic (when the Atlantic was just a narrow channel) and later dispersed to other parts of the world. As both Lavery et al. (2004) and this study indicated that the Western Hemisphere (including the present east Pacific and the Atlantic region) shrimps, *Litopenaeus* and *Farfantepenaeus*, are closely related and that their common ancestors had colonized the New World once during the mid Cretaceous (this study), the hypothesis that *Penaeus s.l.* originated from the Western Hemisphere (Burkenroad, 1934; Pérez Farfante 1969; Van Stemberg 1997) appears unlikely.

Supposing that the *Penaeus* shrimp ancestors originated in the Northwest Tethys Sea, the directions of their dispersal can be controversial. Dall (1990) proposed that the *Penaeus* shrimps had distributed circumglobally before the creation of the Old World Barrier and the closure of Tethys Sea (12-20 MYA) sundered the populations and led to speciation into the Pacific and Atlantic lineages. By showing that the Western Hemisphere shrimps diverged from their Pacific relatives during the mid Cretaceous, our analyses disprove Dall's notion that the Old World Barrier and the closure of Tethys that occurred much later played an important role in *Penaeus* diversification. However our molecular dating results do support that the shrimps should have colonized both the Indo-West Pacific and the Atlantic well before the creation of the Old World Barrier in the late Paleogene. Based on fossil records, during the lower Cretaceous the *Penaeus* ancestor diverged into two lineages. One of them predominantly dispersed eastward into the Tethys Ocean, i.e. the present Indian and Pacific Ocean and established the *Melicertus* clade. The present non-*Melicertus* contains two lineages, one inhabiting the Indo-Pacific region while the other found in the Western Hemisphere. The time when the Western Hemisphere shrimps diverged from their non-*Melicertus* relatives were estimated to be 94 MYA, and this coincided with the widening of the Atlantic Ocean between Europe and North America which appeared to begin in early Cretaceous, and also with the shrinkage of the Tethys Sea, hence restricting gene flow between the Old and New World, that started about 120 MYA (Smith et al., 1994). It is therefore possible that the Western Hemisphere shrimps ancestors colonized the Laurentia along coastal waters when the American continent was still close to the Tethys Sea, and then gradually diverged from their Tethys relatives due to continental drift. Later the closure of the

Tethys Sea and creation of Old World Barrier completely delimited gene flow between oceans. Similar scenario has been suggested in several genera of sturgeon fish (Peng et al., 2007) and needlefish (Banford et al., 2004).

Such a westward dispersal from the East Atlantic to West Atlantic and subsequent East Pacific has been proposed by Rosen (1975). Under Rosen's Eastern Atlantic/Eastern Pacific track model, it can be expected that in a lineage with a pan-Atlantic distribution, the basal taxa should inhabit the East Atlantic. With only limited sampling in *Farfantepenaeus* and *Litopenaeus*, this study could not provide support on this regard. However, results of previous studies that employ 16S and COI gene sequences to reconstruct phylogeny of Penaeidae revealed that the basal species of *Farfantepenaeus* and *Litopenaeus* inhabit East Pacific while the only East Atlantic species *Farfantepenaeus notialis* seemed to have diverged from its West Atlantic sister species only recently (Lavery et al, 2004; Voloch, unpublished data). This implies that the extant Western Hemisphere species diversified from East Pacific to the West Atlantic before the closure of the Isthmus of Panama in 2 MYA, and subsequently crossed the Atlantic Ocean to reach European and African coasts.

Two alternative hypotheses could explain this phylogeographical pattern. One possibility is that, just as aforesaid, the Western Hemisphere shrimps colonized Laurentia and diverged due to continental drift in mid Cretaceous. Some of these founders settled in the west coast of Laurentia before the end Cretaceous catastrophic mass extinction (~65 MYA) devastated their Atlantic ancestors. The Cretaceous-Tertiary (K-T) mass extinction caused extinction of over 80% of

decapod crustaceans (Schweitzer 2001) and gastropods (Sohl, 1987). Our results also suggest that this K-T extinction had profound effects on *Penaeus* shrimps as both of the two lineages in the non-*Melicertus* clade and also the *Melicertus* clade exhibited radiation around 60-66 MYA which seems to correspond to a post extinction recovery of the surviving *Penaeus* ancestral lineages. Be it the case, the *Penaeus* shrimps that inhabited East Pacific might have diversified back to the Atlantic in early Paleogene and this can account for the absence of basal *Farfantepenaeus/Litopenaeus* shrimps in the East Atlantic even though the ancestors of these shrimps might have originated there.

Another plausible explanation is that the ancestors of *Penaeus* shrimps diversified predominantly eastward from the Tethys Sea and did not colonize the Atlantic soon after it opened, as suggested by Lavery et al. (2004). Under this hypothesis a group of founders diverged from their non-*Melicertus* relatives in the West Pacific and crossed the vast (probably half of the globe in Cretaceous epoch) Pacific Ocean to reach North and South America. These founders might have arrived at the America continents by the early Cretaceous and then diversified eastward to the East Atlantic recently. This can explain the lack of relics *Farfantepenaeus* and *Litopenaeus* in the East Atlantic. Crossing the East Pacific Barrier has been documented in only small number of animals, most of which has long pelagic larval stage, including fish, crustaceans, mollusks, echinoderm and hermatypic corals (Briggs, 1974). Although *Penaeus* shrimps have pelagic larval stage, these shrimps require costal estuaries as nurseries to complete their life cycle (Dall, 1990) and with the scarce central Pacific islands to act as stepping stones in the mid Cretaceous, trans-Pacific migration could

have been rather difficult. However, it is impossible to explicitly discern these two hypotheses of colonization routes, until *Penaeus* fossils can be discovered in the mid Atlantic/Pacific to add new insights to this issue.

These divergence time estimations have to be treated with caution. There is disparity in the time of divergence of the Penaeini from the other tribes estimated between this (304 MYA) and the previous chapter (229 MYA, fig 2.4) of the study. On one hand, the use of only one calibration point in this chapter may lead to inaccuracies. On the other hand, the analyses of only a limited number of *Penaeus* species in the previous chapter have led to error in phylogenetic inference (*Penaeus s.l.* was found paraphyletic), which in turn caused miscalculation in divergence time estimations.

3.5 Conclusions

With a large volume of genetic data and substantial taxon sampling, this study has clarified the phylogenetic relationships among the six genera of *Penaeus s.l.* proposed by Pérez Farfante and Kensley (1997) and their Penaeini relatives. While our results strongly support the monophyly of *Penaeus s.l.*, two of the new genera are found paraphyletic. Reverting to the old classification scheme (i.e. a single genus *Penaeus*) is found more proper. Besides, this study also provides novel insights to the divergence times and phylogeography of the *Penaeus* shrimps and these can serve as new hypotheses for further investigations on the evolutionary history of these commercially and ecologically important shrimps.

Chapter 4

General conclusion

This study has presented new insights to the phylogeny of superfamily Penaeoidea and family Penaeidae using DNA sequences of two nuclear protein-coding genes PEPCK and NaK, and also elucidated the phylogenetic relationships among the *Penaeus s.l.* species based on sequences from nuclear protein coding-gene enolase, mitochondrial 12S and 16S rRNA genes, in addition to PEPCK and NaK. The incorporation of these nuclear protein-coding genes in phylogenetic reconstruction has yielded much improved resolutions and statistical supports in the resulting trees when compared to previous molecular analyses that utilized mitochondrial markers only. Nuclear protein-coding genes such as PEPCK, NaK and enolase employed in this study are recommended as core markers for future decapod phylogenetics studies, especially for high level systematics. Using the robust phylogenetic trees this study also, for the first time, estimated the divergence ages of the penaeoid species and contributed to the understanding of the evolutionary history of these shrimps.

The present study has revealed that the penaeoid shrimps constitute two lineages, one composed of the deep-water families Aristeidae, Benthesicymidae and Solenoceridae, while the other included Sicyoniidae and Penaeidae that inhabit shallow-waters. The divergence of these lineages may be caused by differential adaptation to bathymetry and it is believed to have occurred during late Permian

from ancestors inhabiting shallow-waters in Laurentia. The penaeid-like lineage might have diversified in the Triassic, during the recovery period of Permo-Triassic extinction. The aristeid-like lineage might have radiated later in the Jurassic, the time when the Pangea divided. Due to the paraphyly of Penaeidae with Sicyoniidae nested within it and the high level of genetic divergence among the three tribes of Penaeidae, taxonomic revisions are proposed such that the three penaeid tribes may be elevated to the family level in order to be comparable to Sicyoniidae. On the contrary, Benthesicymidae was found so closely associated with Aristeidae that it is questionable whether or not it justifies a family ranking.

Regarding the phylogeny of *Penaeus s.l.*, this study has revealed clear and well-supported phylogenetic relationships among the *Penaeus* shrimps. The results have confirmed that the scheme proposed by Pérez Farfante and Kensley (1997) was not entirely natural. The old scheme, i.e. grouping all 28 species into one *Penaeus* genus, is found more appropriate. In addition, the current study has also provided an estimation of divergence times of the *Penaeus* shrimps. These estimations, together with the phylogenetic relationships reconstructed in this study, have given new insights to hypothesize where and how the ancestors of *Penaeus* shrimps originated and colonized the globe. The ancestors of *Penaeus s.l.* might have emerged in the northwest Tethys Sea during the late Jurassic. In the Cretaceous they might have either colonized both westward to the Atlantic and eastward to the present Indian Ocean, or diversified predominantly westward to achieve the global distribution nowadays.

In conclusion, the use of new nuclear protein-coding markers plus the more traditional mitochondrial markers has not only unambiguously resolved almost all of the phylogenetic relationships among the penaeoid families, the penaeid genera and *Penaeus s.l.* species, but also provided new insights on how these shrimps originated and diversified. To pursuit further understanding of the evolutionary history of these fauna will require new information from fossils, morpho-cladistic analyses and the ecology of these animals.

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